REMARKS

Applicant wishes to thank Examiners Akhavan and Guzo for the courtesy extended to inventor Dr. Baum and the representative, Nancy Vensko, attorney of record, on November 24, 2003. In place of Interview Summary Form PTOL-413, the following summarizes the discussions held at the telephone interview: A prototype version of Claim 8 was discussed. International Preliminary Examination Report (IPER) and Delporte et al., Proc. Natl Acad. Sci. USA 94: 3268 (1997) were shown as exhibits. The claims are free of the prior art. All issues related to 112 first paragraph were discussed. The present response to the outstanding Office Action includes the substance of the Examiner Interview.

A. Disposition of Claims

By this amendment, Applicant has canceled Claims 1-7 without prejudice. Claim 8 has been added. Thus, Claim 8 is pending. This amendment is presented to make explicit what was implicit in the Original Claims. Support is found throughout the Patent Specification, as shown in the following Support Chart. The Patent Specification has been amended to conform the Brief Description of the Drawings with the Detailed Description of the Invention 7:6 - 14:14. No new matter has been added. Reexamination and reconsideration of the application, as amended, are respectfully requested.

Support Chart

Claim Recitation	Support in Specification
a water and salt permeable, porous biodegradable blind end tube having an interior surface	3:7-8, 3:10-11, 10:1-3
matrix components coating said interior surface capable of promoting the formation of a polarized monolayer of salivary gland epithelial cells	3:8-9, 9:18-21, 13:20
a polarized monolayer of salivary gland epithelial cells, said salivary gland epithelial cells disposed on said interior surface and having a phenotype in which said salivary gland epithelial cells express or are transduced to express a water channel protein and ion transport protein so that water and salt is secreted unidirectionally	3:9-10, 3:27-4:12, 12:27-29

B. Compliance with 35 USC 112, Second Paragraph

The Patent Office rejected the claims under 35 USC 112/2 as being indefinite. According to MPEP 2173.02, the claims must be definite when analyzed in light of the Patent Specification. Per the Support Chart, Applicant has conformed the Claim Language to the Patent Specification by use of the terms "water channel protein" and "express or are transduced to express". When analyzed in light of the Patent Specification, the Claim Language is definite.

C. Compliance with 35 USC 112, First Paragraph, Written Description

The Patent Office rejected the claims under 35 USC 112/1 as failing to meet the written description requirement. According to MPEP 2163, the written description may be met through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. Per the Support Chart, Applicant has conformed the Claim Language to the Patent Specification by use of the term "salivary gland epithelial cells". Per the Support Chart, as shown by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, Applicant was in possession of the claimed genus and has met the written description requirement.

D. Compliance with 35 USC 112, First Paragraph, Enablement Requirement

The Patent Office rejected the claims under 35 USC 112/1 as failing to meet the enablement requirement. The test for enablement is whether one skilled in the art could make or use the invention from the disclosure in the patent specification coupled with information known in the art without undue experimentation. *United States v. Telectronics Inc.*, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). Beginning with the disclosure in the patent specification, per the Support Chart, Applicant has conformed the Claim Language to the Patent Specification. Turning to the

state of the art, Declaration of Dr. Baum, attached, testifies on the state of the art as it existed at the time of the effective filing date of this application, 24 February 1999. Based on the disclosure in the patent specification coupled with information known in the art, the conclusion is that one skilled in the art could make or use the invention without undue experimentation as a first generation artificial salivary gland.

Additionally, while evidence of safety in the treatment of humans or regarding the degree of effectiveness will ultimately need to be provided for FDA approval, under MPEP 2107.03 V, FDA approval is not a prerequisite for finding an invention useful within the meaning of the patent laws. Thus, under this provision, it is improper for Office personnel to request evidence of safety in the treatment of humans or regarding the degree of effectiveness. Rather, the relevant inquiry is whether enablement is commensurate in scope with the claims.

Finally, although not authoritative, the opinion of the International Preliminary Examination Authority (IPEA) expressed in International Preliminary Examination Report (IPER), attached, is nonetheless persuasive that the enablement requirement is met. Here, the IPEA takes the position that Baum et al., Annals of the New York Academy of Sciences 875: 294 (June 1999) is novelty-defeating (and, by definition, fully enabling) for the subject matter of the claims. This is because the document reports a conference that was held on 18-22 July 1998, before the priority date of 24 February 1999. Under MPEP 715.01(c), however, an inventor cannot create 102(a) art against himself. Any public disclosure occurred within the one-year grace period. Therefore, a rejection under 35 USC 102(a) cannot stand. While not prior art date-wise (under U.S. patent law), Baum et al. June 1999 is nonetheless fully enabling for the subject matter of the claims (per the opinion of the IPEA). Yet Baum et al. June 1999 corresponds to the priority document (and benefit of priority is not in dispute with either the IPEA or the USPTO). Thus the priority document is fully enabling for the subject matter of the claims (per the opinion of the IPEA). The conclusion of this International Authority is an unbiased third-party opinion that is persuasive that the enablement requirement is met.

CONCLUSION

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding rejections are respectfully requested. Allowance

of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 11/26/03

By: _____

Nancy W. Vensko
Registration No. 36,298
Attorney of Record
Customer No. 20,995
(805) 547-5585

AMEND
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NIH 96.001C1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Baum et al.
Appl. No.	:	09/938,706
Filed	:	August 23, 2001
For	:	ARTIFICIAL SALIVARY GLAND
Examiner	:	Akhavan, Ramin
Group Art Unit	:	1636

DECLARATION OF BRUCE J. BAUM

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I am one of the inventors of the above-identified application.

2. A true and correct copy of my Curriculum Vitae is attached as Exhibit 1.

3. This declaration is to comment on the state of the art as it existed at the time of the effective filing date of this application, 24 February 1999.

4. Although it is possible to transplant mammalian salivary glands (Eid et al., Transplantation 64: 679 (1997), of record, re-attached), due to clinical inadequacy of this option, the problem in the prior art was the lack of an artificial salivary gland. Each year in the U.S., about 30,000 individuals undergo therapeutic ionizing radiation to their salivary glands during radiation therapy for head and neck malignancies. Patients whose salivary glands are thus rendered hypofunctional suffer from rampant dental caries (decay), frequent mucosal infections

(such as oral Candidiasis), dysphagia (swallowing difficulties), as well as considerable pain and discomfort. There was no conventional effective therapy for this condition.

5. Nonetheless, pre-filing date studies of elements of the artificial salivary gland constituted proof of principle experiments.

6. Mooney et al., Cell Transplant. 3: 203 (1994), of record, re-attached, reported the construction of a tubular and porous biodegradable substrate which promoted vascularization following implantation.

7. Royce et al., Differentiation 52: 247 (1993), of record, re-attached, reported the differentiation of a salivary duct cell line on a reconstituted basement membrane.

8. Delporte et al., Proc. Natl Acad. Sci. USA 94: 3268 (1997), of record, re-attached, reported the construction of a recombinant adenovirus encoding human aquaporin-1 (hAQP1), the archetypal water channel. This virus directed hAQP1 expression *in vitro* in several epithelial cell lines (Fig. 1) and *in vivo* in salivary gland (Fig. 5). In polarized epithelial cell monolayers, hAQP1 was localized in the apical and basolateral plasma membranes (Fig. 2). Fluid movement across monolayers infected by recombinant adenovirus encoding hAQP1 in response to an osmotic gradient was about 4-fold that seen with uninfected monolayers or monolayers infected by a control virus (Fig. 3). When the virus was administered to salivary glands that had been irradiated and rendered hypofunctional, salivary flow rate was dramatically increased (Fig. 4).

9. Due to the reason that adenovirus-mediated gene transfer leads to transient transgene expression and a significant host immune response, Braddon et al., Human Gene Therapy 9: 2777 (1998), newly attached, investigated use of recombinant adenoassociated viruses, which feature no association with any disease and an ability to integrate into the host

genome, and reported adenoassociated virus-mediated transfer of a functional water channel into salivary epithelial cells *in vitro* and *in vivo*.

10. Voutetakis et al., submitted, reported that administration of a recombinant adenoassociated viral vector encoding human erythropoietin (hEPO) directly to salivary gland resulted in serum hEPO levels that reached maximum proportions 8-12 weeks after gene delivery and remained relatively stable for 54 weeks.

11. Because the artificial salivary gland would actually operate within the parameters disclosed in the patent specification, as shown by pre-filing date studies of elements of the artificial salivary gland constituting proof of principle experiments (and confirmed by the post-filing art of Voutetakis et al., submitted), the invention defined in the claims solves the problem in the prior art caused by clinical inadequacy of other options by offering a therapy that could be expected to last up to one year for patients whose salivary glands are hypofunctional and have no hope for conventional effective therapy for this condition.

12. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Respectfully submitted,

Dated: November 26, 2003

By: Bruce Baum
Bruce J. Baum, D.M.D., Ph.D.

EXHIBIT 1

November 25, 2003

CURRICULUM VITAE

NAME: Bruce Jay Baum

OFFICE ADDRESS: GTTB, NIDCR, NIH
Building 10, Room 1N-113
10 Center Drive, MSC 1190
Bethesda, MD 20892-1190

OFFICE PHONE: (301) 496-1363
FAX: (301) 402-1228
E-MAIL: bbaum@dir.nidcr.nih.gov

DATE and PLACE OF BIRTH: October 28, 1945; Lynn, Massachusetts

CITIZEN: United States

EDUCATION:

1967 - B.A. University of Virginia, Charlottesville, VA

1971 - D.M.D. Tufts University, Boston, MA

1974 - Ph.D. Boston University, Boston, MA

BOARD CERTIFICATION:

1971 - National Board of Dental Examiners

LICENSURE:

Massachusetts, 1971 (currently inactive)

Maryland, 1976

BRIEF CHRONOLOGY OF EMPLOYMENT:

1974 - 1976	Naval Dental Research Officer, Biochemistry and Oral Bacteriology Division, Dental Sciences Department, Naval Medical Research Institute, Bethesda, MD
1976 - 1978	Staff Investigator, Pulmonary Branch, National Heart, Lung and Blood Institute, Bethesda, MD
1978 - 1981	Senior Investigator, Laboratory of Molecular Aging, Gerontology Research Center, National Institute on Aging, Baltimore, MD
1982 - 1996	Clinical Director and Chief, Clinical Investigations and Patient Care Branch, National Institute of Dental Research, Bethesda, MD
1996 – present	Chief, Gene Therapy and Therapeutics Branch, National Institute of Dental and Craniofacial Research, Bethesda, MD

MILITARY SERVICE:

1974 - 1976 U.S. Navy

PROFESSIONAL ORGANIZATIONS:

American Academy of Oral Medicine

American Association for the Advancement of Science

American Dental Association

American Dental Education Association

American Society for Biochemistry and Molecular Biology

American Society of Gene Therapy

American Physiological Society

International Association for Dental Research

HONORARY SOCIETIES:

Alpha Epsilon Delta

Omicron Kappa Upsilon

Sigma Xi

HONORS and OTHER SPECIAL SCIENTIFIC RECOGNITION:

B.A. with distinction, University of Virginia, 1967

Clarence Pettengill Fellowship, Tufts University, 1970

Alumni Award for Social Dentistry, Tufts University, 1971

U.S. Public Health Service Commendation Medal, 1982, 1988

Lady Davis Fellowship and Visiting Professorship, Hebrew University, 1984

Carl A. Schlack Award, Association of Military Surgeons of the U.S., 1987

Wellcome Visiting Professorship, 1988, University of Pittsburgh

Researcher of the Year, Salivary Research Group, IADR, 1988

Visiting Professorship, University of Liverpool, 1988

Visiting Professorship, Tel-Aviv University, 1989

Goldstein Visiting Professorship, Hebrew University, 1990

Distinguished Lecturer in Oral Biology, SUNY Buffalo, 1990

Fellow, American Association for the Advancement of Science, 1992

Salivary Research Award, IADR, 1993

Samuel Charles Miller Award, American Academy of Oral Medicine, 1993

Dr. J. Murray Gavel Clinical Research Lectureship, Forsyth Dental Center, Boston, MA, 1994

U.S. Public Health Service Citation, 1994

Geriatric Oral Research Award, IADR, 1995

Visiting Professorship, Tokyo Dental College, 1996 - present

NIH Senior Biomedical Research Service, 1996

Columbia University Birnberg Research Award, 1997

NIDR Director's Exemplary Service Award, 1997, 2000

Visiting Professorship, Harvard School of Dental Medicine, 1997

Honorary Professorship, Capital University of Medical Sciences, Beijing, PRC, 1999

Jack Lewin Epstein Memorial Lectureship, Hebrew University, 2000

Norton Ross Award, American Dental Association, 2000

Visiting Professorship, Tokyo Medical and Dental University, 2001

Leo M. Sreebny Visiting Scholar, SUNY-Stony Brook, 2002

Isidore Cabakoff Lectureship, Hebrew University, 2002

New York University, Distinguished Scientist Award, 2003

RESEARCH INTERESTS:

Aging and oral physiology/oral health
Regulation of salivary gland secretion
Pathogenesis of salivary gland disorders
Clinical applications of gene transfer technology

OTHER ACTIVITIES:

1973 - 1978	Abstractor, Oral Research Abstracts, Biochemistry Section
1976 - 1993	Editorial Review Board, Journal of Dental Research
1979 - present	Ad Hoc Reviewer or Consultant for agencies such as Veterans Administration Medical Research Service; National Science Foundation; National Institutes of Health, Extramural Grants; Medical Research Council of Canada; Israel Academy of Sciences and Humanities; Canadian Cystic Fibrosis Foundation; Israel Science Foundation; Wellcome Trust (UK); NATO Scientific Exchange Program; and for numerous scientific journals

1980 - 1983	Secretary-Treasurer, Vice-President, President, Washington Section, American Association for Dental Research
1980 - 1982	Program Officer, Salivary Research Group, International Association for Dental Research
1980 - 1981	Ad Hoc Advisor, 1981 White House Conference on Aging
1981 - 1984	Member, Long Range Planning Committee, American Association for Dental Research
1981 - 1991	Associate Editor for Basic Science, Gerodontology
1982	Visiting Scientist, Department of Physiological Chemistry, Johns Hopkins University, Baltimore, MD
1983 - 1990	NIDR representative, NIH Cystic Fibrosis Co-ordinating Committee
1983 - 1985	Chairman, Publication Committee, Journal of Dental Research
1984 - 1985	Member, Project Advisory Panel, NIDR/NIA/VA Collaborative Project on Oral Health in the Elderly
1985 - 1987	Editorial Board, Dysphagia
1985 - 1989	Member, Long Range Planning Committee, American Association for Dental Research
1986 - 1988	Chairman, National Institute on Aging, Clinical Research Subpanel
1986 - 1989	Member, Gies Award Committee, American Association for Dental Research
1987 - 1991	Member-at-Large, Section R (Dentistry) American Association for the Advancement of Science
1987 - 1995	Chair, Clinical Center Medical Board Tenure Promotion Committee
1987 - 1992	Associate Editor, Dysphagia

1987 - 1988	Programming Committee, American Society for Biochemistry and Molecular Biology
1988 - 1992	Editorial Board, Journal of Gerontology: Medical Sciences
1988	Chairman, Section 4, ADA-World Workshop on Oral Medicine
1988	Consultant, Geriatric Education Project, American Association of Dental Schools
1989	Member, Institute of Medicine, Clinical Liason Team: A National Research Agenda on Aging.
1989	Member, National Institute of Deafness and other Communicative Disorders Task Force to Develop a National Strategic Research Plan
1989 - 1994	Consultant, Commission on Oral Health, Research and Epidemiology, Federation Dentaire Internationale
1989 - 1990	Chairman, ad hoc Work Group on the Future Scope of NIDR Research Activities
1991 - 1994	Chairman, International Conference of Oral Biology Planning Committee, International Association for Dental Research
1991 - 2002	Member, Planning Committee, World Workshops on Oral Medicine
1991 - 2002	Editorial Board, Oral Surgery, Oral Medicine and Oral Pathology (Oral Medicine Section)
1992 - 1997	Editorial Board, Dysphagia
1992	Guest Scientist, Laboratory of Cellular Metabolism, National Heart, Lung and Blood Institute, Bethesda, MD
1993 - 1994	Chairman, NIDR-IRP Internal Advisory Committee
1993	Member, Oral Health 2000 Scientific Advisory Task Force
1994 - 1998	AADR representative to AAAS (Section N-Medical Sciences)
1994 - 1996	Special Consultant in Dental Education, Indiana University School of Dentistry

1994 - 1996	Editorial Board, Journal of Dental Research
1994 - 1996	Chair-Elect, then Chair, Section on Dentistry, American Association for the Advancement of Science
1995	Member, External Review Committee, Faculty of Dentistry, University of Sydney, Australia
1995 - 1996	Chair, Medical Board Subcommittee on Training and Career Paths
1995 - 1996	Member, Steering Committee, NIDR Strategic Planning Process
1995 – 1997	Member, NIH Committee on Scientific Conduct and Ethics
1996	Member, NIDR-DIR Reorganization Committee
1996 – present	Clinical Professor, Department of Oral Health Care Delivery, University of Maryland
1996 - present	Associate Editor, Clinical Sciences, European Journal of Dental Education
1996 - present	Board of Tutors, NIH Clinical Research Training Program
1997 - present	Member, NIH Senior Biomedical Research Service Policy Board
1997 – 2000	Member, NIH Clinical Research Revitalization Committee
1998 – 2001	Editorial Board, Journal of Dental Research
1999	Chair, Salivary Glands and Saliva Gordon Research Conference
1999 – present	Member, Admissions Committee, NIH-Duke Master of Health Sciences in Clinical Research program
1999 – present	Senior Editor, Oral Diseases
1999	Member, NIDCR Blue Ribbon Panel on Research Training and Career Development
1999	Member, External Review Panel, University of Pittsburgh School of Dental Medicine
1999 - present	Member, Board of Overseers, Tufts University School of Dental Medicine

1999	Member, NIH Clinical Center Revitalization Steering Committee
1999 – 2002	NIDCR Liason, NIH Office of Recombinant DNA Activities
1999 – 2002	Member, Scientific Advisory Board, Genteric, Inc, Alameda, CA
1999 – 2001	Member, Future of Dentistry Committee, American Dental Association
2000	Member, External Review Panel, Columbia University School of Dental and Oral Surgery
2000-present	Consultant, Inspire Pharmaceuticals, Durham, NC
2000- 2001	Chair, Section 5.3, DentEdEvolves, Association for Dental Education in Europe
2001	Member, External Review Committee, School of Dentistry, Tokushima University, Japan
2002	Member, External Review Committee, School of Dentistry, University of Southern California
2002-present	Member, Scientific Advisory Board, Dentigenix, LLC, Kirkland, WA
2003-present	Member, Scientific Advisory Board, Harvard School of Dental Medicine, Boston, MA

BIBLIOGRAPHY

Peer-reviewed publications:

1. Baum, B.J.: Medicaid and the practicing dentist. J. Mass. Dent. Soc. 18: 30-31, 1969.

2. Cohen, M.M., Arvystas, M.G. and Baum, B.J.: Occlusal disharmonies in trisomy G. Am. J. Orthod. 58: 367-372, 1970.

3. Baum, B.J. and Cohen, M.M.: Patterns of size reduction in hypodontia. J. Dent. Res. 50: 779. 1971.

4. Baum, B.J. and Cohen, M.M.: Agenesis and tooth size in the permanent dentition. Angle Orthod. 41: 100-102, 1971.

5. Baum, B.J. and Cohen, M.M.: Studies on agenesis in the permanent dentition. Am. J. Phys. Anthropol. 35: 125-128, 1971.

6. Baum, B.J., Margolies, M.R., Doku, M.C. and Murnane, T.W.: The effect of α-chymotrypsin on wound healing in hamsters. Oral Surg. 33: 484-489, 1972.

7. Baum, B.J. and Cohen, M.M.: Decreased odontometric sex differences in individuals with dental agenesis. Am. J. Phys. Anthropol. 38: 739-742, 1973.

8. Baum, B.J., Troxler, R.F., Kagan, H.M., Grasso, J.A., Faris, B. and Franzblau, C.: Incorporation of L-azetidine-2-carboxylic acid into hemoglobin in rabbit reticulocytes. Biochem. Biophys. Res. Commun. 43: 1350-1356, 1973.
9. Baum, B.J., Franzblau, C. and Kagan, H.M.: Isolation and partial characterization of collagen from newt tail. Comp. Biochem. Physiol. 47: 805-813, 1974.

10. Baum, B.J., Faris, B. and Franzblau, C.: Further studies on newt-tail collagen: description of lysine derived crosslinks. Comp. Biochem. Physiol. 51: 51-55, 1975.

11. Baum, B.J., Johnson, L.S., Franzblau, C. and Troxler, R.F.: Incorporation of L-azetidine-2-carboxylic-acid into hemoglobin in rabbit reticulocytes in vitro. J. Biol. Chem. 250: 1464-1471, 1975.

12. Franzblau, C., Schmid, K., Faris, B., Beldekas, J., Gavin, P., Kagan, H.M. and Baum, B.J.: The interaction of collagen with α_1-acid glycoprotein. Biochim. Biophys. Acta 427: 302-314, 1976.

13. Polyzois, S., Baum, B.J., Bowen, W.H. and Longton, R.W.: Differences in the pH activity profile of human and monkey salivary lysozyme. J. Dent. Res. 55: 1137, 1976.

14. Baum, B.J., Bird, J.L., Millar, D.B. and Longton, R.W.: Studies on histidine rich polypeptides from human parotid saliva. Arch. Biochem. Biophys. 177: 427-436, 1976.

15. Baum, B.J., Bird, J.L., Millar, D.B. and Longton, R.W.: Isolation and partial characterization of an histidine-rich-polypeptide from parotid saliva of the monkey, Macaca nemestrina. Comp. Biochem. Physiol. 56A: 115-120, 1977.

16. Baum, B.J., Bird, J.L. and Longton, R.W.: Polyacrylamide gel electrophoresis of human salivary histidine-rich-polypeptides. J. Dent. Res. 1115-1118, 1977.

17. Bird, J.L., Baum, B.J., Makinen, K.K., Bowen, W.H. and Longton, R.W.: Xylitol associated changes in amylase and protein content of monkey parotid saliva. J. Nutr. 107: 1763-1767, 1977.

18. Baum, B.J., Bird, J.L. and Longton, R.W.: Histidine-rich-polypeptides in parotid saliva are not nuclear histones. Arch. Oral Biol. 22: 455-456, 1977.

19. Baum, B.J., Ellison, S.A. and Levine, M.J.: Immunochemical detection of human saliva histidine-rich-polypeptides. Arch. Oral Biol. 22: 457-459, 1977.

20. Baum, B.J., Bird, J.L. and Longton, R.W.: Evidence that the histidine-rich-polypeptides of human parotid saliva are secretory products of the parotid gland. J. Dent. Res. 56: 877, 1977.

21. Baum, B.J., McDonald, J.A. and Crystal, R.G.: Metabolic fate of the major cell surface protein of normal human fibroblasts. Biochem. Biophys. Res. Commun. 79: 8-15, 1977.

22. Baum, B.J., Moss, J., Breul, S.D. and Crystal, R.G.: Association in normal human fibroblasts of elevated levels of adenosine 3':5'- monophosphate with a selective decrease in collagen production. J. Biol. Chem. 253: 3391-3394, 1978.

23. Bienkowski, R.S., Baum, B.J. and Crystal, R.G.: Fibroblasts degrade newly synthesized collagen within the cell before secretion. Nature 276: 413-416, 1978.

24. McDonald, J.A., Baum, B.J., Rosenberg, D.M., Kelman, J.A., Brin, S.C. and Crystal, R.C.: Destruction of a major extracellular adhesive glycoprotein (fibronectin) of human fibroblasts by neutral protease from polymorphonuclear leukocyte granules. Lab. Invest. 40: 350-357, 1979.

25. Steinmann, B.U., Martin, G.R., Baum, B.J. and Crystal, R.G.: Synthesis and degradation of collagen by skin fibroblasts from controls and from patients with osteogenesis imperfecta. FEBS Lett. 101: 269-272, 1979.

26. Baum, B.J. and Wright, W.E.: Demonstration of fibronectin as a major extracellular protein of human gingival fibroblasts. J. Dent. Res. 59: 631-637, 1980.

27. Baum, B.J., Moss, J., Breul, S.D., Berg, R.A. and Crystal, R.G.: Effect of cyclic AMP on the intracellular degradation of newly synthesized collagen. J. Biol. Chem. 255: 2843-2847, 1980.

28. Ito, H., Hoopes, M.T., Roth, G.S. and Baum, B.J.: Adrenergic and cholinergic mediated glucose oxidation by rat parotid gland acinar cells during aging. Biochem. Biophys. Res. Commun. 98: 275-282, 1981.

29. Cole, M.F., Hsu, S.D., Baum, B.J., Bowen, W.F., Sierra, L.I., Aquirre, M. and Gillespie, G.: Specific and nonspecific immune factors in dental plaque fluid and saliva from young and old populations. Infect. Immun. 31: 998-1002, 1981.

30. Baum, B.J. Current research on aging and oral health. Special Care in Dentistry 1:105-109, 1981.

31. Baum, B.J., Colpo, F.T. and Filburn, C.R.: Rat parotid gland cyclic AMP-dependent protein kinase: characterization and relationship to exocrine secretion. Arch. Oral Biol. 26: 333-337, 1981.

32. Ito, H., Baum, B.J. and Roth, G.S.: β-Adrenergic regulation of rat parotid gland exocrine protein secretion during aging. Mech. Ageing Develop. 15: 177-188, 1981.

33. Kuyatt, B.L. and Baum, B.J.: Characteristics of submandibular glands from young adult and aged rats. J. Dent. Res. 60: 936-941, 1981.

34. Baum, B.J.: Evaluation of stimulated parotid saliva flow rate in different age groups. J. Dent. Res. 60: 1292-1296, 1981.

35. Baum, B.J. and Kuyatt, B.L.: Protein production and release by dispersed rat submandibular gland cells in vitro after adrenergic stimulation. Life Sci. 29: 1143-1151, 1981.

36. Baum, B.J.: Characteristics of participants in the oral physiology component of the Baltimore longitudinal study of aging. Community Dent. Oral Epidemiol. 9: 128-134, 1981.

37. Baum, B.J., Freiberg, J.M., Ito, H., Roth, G.S. and Filburn, C.R.: β-Adrenergic regulation of protein phosphorylation and its relationship to exocrine secretion in dispersed rat parotid gland acinar cells. J. Biol. Chem. 256: 9731-9736, 1981.

38. Berg, R.A., Moss, J., Baum, B.J. and Crystal, R.G.: Regulation of collagen production by the β-adrenergic system. J. Clin. Invest. 67: 1457-1462, 1981.

39. Ito, H., Hoopes, M.T., Baum, B.J. and Roth, G.S.: K+ release from rat parotid cells is an α_1-adrenergic mediated event. Biochem. Pharmacol., 31: 567-573, 1982.

40. Uchida, T., Ito, H., Baum, B.J. and Roth, G.S., Filburn, C.R. and Sacktor, B.: Alpha-1-adrenergic stimulation of phosphatidylinositol-phosphatidic acid turnover in rat parotid gland. Mol. Pharmacol. 21: 128-132, 1982.

41. Weiffenbach, J.M., Baum, B.J. and Burghauser, R.: Taste thresholds: quality specific variation with human aging. J. Gerontol. 37: 372-377, 1982.

42. Baum, B.J., Levine, R.L., Kuyatt, B.L. and Sogin, D.B.: Rat parotid gland amylase: evidence for alterations in an exocrine protein with increased age. Mech. Ageing Develop. 19: 27-35, 1982.

43. Baum, B.J., Kousvelari, E.E. and Oppenheim, F.G.: Exocrine protein secretion from human parotid glands during aging: stable release of the acidic proline-rich proteins. J. Gerontol. 37: 392-395, 1982.

44. Kuyatt, B.L. and Baum, B.J.: Characteristics of sublingual glands from young adult and aged rats. Gerodontology 1: 5-9, 1982.

45. Ito, H., Baum, B.J., Uchida, T., Hoopes, M.T., Bodner, L., and Roth, G.S.: Modulation of rat parotid cell alpha adrenergic responsiveness at a step subsequent to receptor activation. J. Biol. Chem. 257: 9532-9538, 1982.

46. Baum, B.J. and Bodner, L.: Aging and oral motor function: Evidence for altered performance among older persons, J. Dent. Res. 62: 2-6, 1983.

47. Bodner, L., Qwarnstrom, E., Omnell, K-A, Hand, A.R. and Baum, B.J.: Rat submandibular gland secretion: a bilateral and longitudinal comparative study. Comp. Biochem. Physiol. 74A: 829-831, 1983.

48. Bodner, L., Hoopes, M.T., Gee, M., Ito, H., Roth, G.S. and Baum, B.J.: Multiple transduction mechanisms are likely involved in calcium-mediated exocrine secretory events in rat parotid cells. J. Biol. Chem. 258: 2774-2777, 1983.

49. Baum, B.J. and Kuyatt, B.L.: Protein production and processing in young adult and aged rat submandibular gland cells.: Mech. Ageing Develop. 23: 123-136, 1983.

50. Bodner, L. and Baum, B.J.: Submandibular gland secretory function in young adult and aged rats. Comp. Biochem. Phsiol. 77A: 235-238, 1984.

51. Bodner, L., Kuyatt, B.L., Hand, A.R. and Baum, B.J.: Rat parotid cell function in vitro following X-irradiation in vivo. Rad. Research. 97: 386-395, 1984.

52. Gee, M.V., Baum, B.J. and Roth, G.S.: Stimulation of parotid glucose oxidation: Role of $alpha_1$-adrenergic receptors and calcium mobilization. Biochem. Pharmacol. 32: 3351-3354, 1983.

53. Kousvelari, E.E., Kusiak, J.W., Hand, A.R., Pitha, J. and Baum, B.J.: Bromoacetylalprenolomenthane: A potent irreversible antagonist of β-adrenergic elicited protein exocytosis in rat parotid cells. J. Pharmacol. Exp. Thera. 227: 238-243, 1983.

54. Kousvelari, E.E., Grant, S.R. and Baum, B.J.: Dolichyl phosphate supplementation increases N-linked protein glycosylation in rat parotid acinar cells without increasing glycoprotein secretion. Exp. Cell Res. 149: 271-276, 1983.

55. Mistretta, C.M. and Baum, B.J.: Quantitative study of taste buds in fungiform and circumvallate papillae of young and aged rats. J. Anat. 138: 323-332, 1984.

56. Kousvelari, E.E., Grant, S.R. and Baum, B.J.: β-Adrenergic receptor regulation of N-linked protein glycosylation in rat parotid acinar cells. Proc. Natl. Acad. Sci. USA 80: 7146-7150, 1983.

57. Baum, B.J., Costa, P.T., Jr. and Izutsu, K.T.: Alterations in sodium handling by human parotid glands during aging: failure to support a simple two stage secretion model. Amer. J. Physiol. 246: R35-R39, 1984.

58. Shawker, T.H., Sonies, B., Stone, M. and Baum, B.J.: Real time ultrasound visualization of tongue movement during swallowing. J. Clin. Ultrasound 11: 485-490, 1983.

59. Sonies, B.C., Baum, B.J. and Shawker, T.H.: Tongue motion in elderly adults: initial in situ observations. J. Gerontol. 39: 279-283, 1984.

60. Qwarnstrom, E., Bodner, L., Baum, B.J., Hand A. and Omnell, K.A.: Saliva from the rat submandibular gland after retrograde infusion of radiographic contrast media. J. Dent. Res. 63: 614-617, 1984.

61. Baum, B.J., Kuyatt, B.L. and Takuma, T.: Adrenergic regulation of protein synthesis in rat submandibular salivary gland cells. Arch. Oral Biol. 29: 499-502, 1984.

62. Shawker, T.H., Sonies, B., Hall, T.E. and Baum, B.J.: Ultrasound analysis of tongue, thyroid and larynx activity during swallowing. Invest. Radiol. 19: 82-86, 1984.

63. MacKay, B.J., Pollock, J.J., Iacono, V.J. and Baum, B.J.: Isolation of milligram quantities of a group of histidine-rich polypeptides from human parotid saliva. Infect. Immun. 44: 688-694, 1984.

64. Kousvelari, E.E., Grant, S.R., Banerjee, D.K., Newby, M.J. and Baum, B.J.: Cyclic AMP mediates β-adrenergic-induced increases in N-linked protein glycosylation in rat parotid acinar cells. Biochem. J. 222: 17-24, 1984.

65. Heft, M. and Baum, B.J.: Unstimulated and stimulated parotid salivary flow rate in different aged individuals. J. Dent. Res. 63: 1182-1185, 1984.

66. Takuma, T., Kuyatt, B.L. and Baum, B.J.: α_1-Adrenergic inhibition of protein synthesis in rat submandibular cells. Amer. J. Physiol. 247: G284-G289, 1984.

67. Takuma, T. and Baum, B.J.: Na+-dependent transport of α-aminoisobutyrate in isolated basolateral membrane vesicles from rat parotid glands. Biochim. Biophys. Acta. 812: 453-459, 1985.

68. Baum, B.J., Bodner, L., Fox, P.C., Izutsu, K.T., Pizzo, P.A. and Wright, W.E. Therapy-induced dysfunctions of salivary glands: implications for oral health. Special Care in Dentistry. 5: 274-277, 1985.

69. Baum, B.J., Kuyatt, B.L., Helman, Y., Gee, M.V. and Roth, G.S.: Alpha1-adrenergic responsiveness of young adult and aged rat submandibular cells in vitro. Comp. Biochem. Physiol. 81C: 121-124, 1985.

70. Banerjee, D.K., Kousvelari, E.E. and Baum, B.J.: β-Adrenergic activation of glycosyltransferases in the dolichylmonophosphate-linked pathway of protein-N-glycosylation. Biochem. Biophys. Res. Commun. 126: 123-129, 1985.

71. Takuma, T., Kuyatt, B.L. and Baum, B.J.: Calcium transport mechanisms in basolateral plasma membrane-enriched vesicles from rat parotid gland. Biochem. J. 227: 239-245, 1985.

72. Fox, P.C., van der Ven, P.F., Sonies, B.C., Weiffenbach, J.M. and Baum, B.J.: Xerostomia: evaluation of a symptom with increasing significance. J. Amer. Dent. Assoc. 110: 519-525, 1985.

73. Helman, J., Roth, G.S. and Baum, B.J.: Adrenergic agonist induced Ca^2+ fluxes in rat parotid cells are not Na-dependent. Biochem. J. 230: 313-320, 1985.

74. Weiffenbach, J.M., Cowart, B.J. and Baum, B.J.: Taste intensity perception in aging. J. Gerontol. 41: 460-468, 1986.

75. Kuyatt, B.L., Roth, G.S. and Baum, B.J.: Analogs of cyclic AMP stimulate glucose oxidation by rat submandibular gland cells in vitro. Arch. Oral Biol. 30: 701-703, 1985.

76. Bodner, L. and Baum, B.J.: Characteristics of stimulated parotid gland secretion in the aging rat. Mech. Ageing Develop. 31: 337-342, 1985.

77. Tabak, L.A., Mirels, L., Monte, L.D., Ridall, A.L., Levine, M.J., Loomis, R.E., Lindauer, F., Reddy, M.S. and Baum, B.J. Isolation and characterization of a mucin-glycoprotein from rat submandibular glands. Arch. Biochem. Biophys. 242: 383-392, 1985.

78. Grant, S.R., Kousvelari, E.E., Banerjee, D.K. and Baum, B.J. β-Adrenergic stimulation alters oligosaccharide pyrophosphoryl dolichol metabolism in rat parotid acinar cells. Biochem. J. 231: 431-438, 1985.

79. Fox, P.C., Bodner, L., Bowers, M.R. and Baum, B.J. Uptake and secretion of technetium pertechnetate by the rat parotid gland. Comp. Biochem. Physiol. 83A: 579-584, 1986.

80. Scott, J., Bodner, L. and Baum, B.J. Assessment of age-related changes in submandibular and sublingual salivary glands of the rat using stereological analysis. Arch. Oral Biol. 31: 69-71, 1986.

81. Scott, J. and Baum, B.J. Involvement of cyclic AMP and calcium in exocrine protein secretion induced by vasoactive intestinal polypeptide in rat parotid cells. Biochim. Biophys. Acta. 847: 255-262, 1986.

82. Fox, P.C., van der Ven, P.F., Baum, B.J. and Mandel, I.D. Pilocarpine for the treatment of xerostomia associated with salivary gland dysfunction. Oral Surg. 61: 243-248, 1986.

83. Gee, M.V., Ishikawa, Y., Baum, B.J. and Roth, G.S. Impaired adrenergic stimulation of rat parotid cell glucose oxidation during aging: the role of calcium. J. Gerontol. 41: 331-335, 1986.

84. van der Ven, P.F., Takuma, T. and Baum, B.J. Chlorpromazine inhibition of muscarinic-cholinergic responses in the rat parotid gland. J. Dental Res. 65: 382-386, 1986.

85. Shiraki, M., Gee, M.V., Baum, B.J. and Roth, G.S. Parathyroid hormone stimulates phosphate efflux through an apparently cAMP independent process in rat parotid cell aggregates. Endocrinology 118: 2009-2015, 1986.

86. Fox, P.C. and Baum, B.J. Isolation of HTLV-III virus from saliva in AIDS (letter). New Eng. J. Med. 314: 1387, 1986.

87. Helman, J., Kuyatt, B.L., Takuma, T., Seligmann, B. and Baum, B.J. ATP-dependent calcium transport in rat parotid basolateral membrane vesicles. Modulation by agents which elevate cyclic AMP. J. Biol. Chem. 261: 8919-8923, 1986.

88. Weiffenbach, J.M., Fox, P.C. and Baum, B.J. Taste and salivary function. Proc. Natl. Acad. Sci. USA 83: 6103-6106, 1986.

89. Kawaguchi, M., Turner, R.J. and Baum, B.J. ^{36}Cl and ^{86}Rb Uptake in rat parotid acinar cells. Arch. Oral Biol. 31: 679-683, 1986.

90. Turner, R.J., George, J.N. and Baum, B.J. Evidence for a $Na^+/K^+/Cl^-$ cotransport system in basolateral membrane vesicles from the rabbit parotid. J. Membr. Biol. 94: 143-152, 1986.

91. Baum, B.J. and Shteyer, A. Characteristics of a neutral amino acid transport system (system A) in osteoblastic rat osteosarcoma cells. Exp. Cell Res. 169: 453-457, 1987.

92. Kousvelari, E.E., Fox, P.C. and Baum, B.J. Regulatory aspects of N-linked glycoproteins. J. Dent. Res. 66: 552-556, 1987.

93. Kousvelari, E.E., Banerjee, D.K., Murty, L. and Baum, B.J. N-Linked protein glycosylation in the rat parotid gland during aging. Mech. Ageing Develop. 42: 173-181, 1988.

94. Kousvelari, E.E., Banerjee, D.K., Grant, S.R. and Baum, B.J. β-Adrenoreceptor activation modulates oligosaccharide processing in an exocrine secretory glycoprotein of rat parotid cells. Arch. Oral Biol., 33: 115-120, 1988.

95. Helman, J., Kusiak, J.W., Pitha, J. and Baum, B.J.. Inhibition of α_1-adrenergic responsiveness in intact cells by a new, irreversible receptor antagonist. Biochem. Biophys. Res. Commun. 142: 403-409, 1987.

96. Hughes, C.V., Baum, B.J., Fox, P.C., Marmary, Y., Yeh, C-K and Sonies, B.C. Oral-pharyngeal dysphagia: a common sequela of salivary gland dysfunction. Dysphagia

97. Helman, J., Turner, R.J., Fox, P.C. and Baum, B.J. $^{99m}Tc^-$ Pertecnetate uptake in parotid acinar cells by the $Na^+/K^+/Cl^-$ co-transport system. J. Clin. Invest. 79: 1310-1313, 1987.

98. Melvin, J.E., Kawaguchi, M., Baum, B.J. and Turner, R.J. A muscarinic agonist - stimulated chloride efflux pathway is associated with fluid secretion in rat parotid acinar cells. Biochem. Biophys. Res. Commun. 145: 754-759, 1987.

99. Marmary, Y., Fox, P.C. and Baum, B.J. Fluid secretion rates from mouse and rat parotid glands are markedly different following pilocarpine stimulation. Comp. Biochem. Physiol. 88A: 307-310, 1987.

100.Fox, P.C., Heft, M.W., Herrera, M., Bowers, M.R., Mandel, I.D. and Baum, B.J. Secretion of anti-microbial proteins from the parotid glands of different aged healthy persons. J. Gerontol. 42: 466-469, 1987.

101.Shiraki, M., Ishikawa, Y., Baum, B.J. and Roth, G.S. Effects of aging on parathyroid hormone stimulated ionic fluxes in rat parotid cell aggregates. Mech. Ageing. Develop., 40: 215-222, 1987.

102.Fox, P.C., Busch, K.A. and Baum, B.J. Subjective reports of xerostomia and objective measures of salivary gland performance. J. Amer. Dent. Assoc. 115: 581-584 1987.

103.Banerjee, D.K., Kousvelari, E.E. and Baum, B.J. cAMP-mediated protein phosphorylation of. microsomal membranes increases mannosylphosphodolichol synthase activity. Proc. Natl. Acad. Sci. USA 84: 6389-6393, 1987

104.Helman, J., Ambudkar, I.S. and Baum, B.J. Adrenoreceptor mobilization of calcium in rat submandibular cells. Eur. J. Pharmacol. 143: 65-72, 1987.

105.Ambudkar, I.S., Kuyatt, B.L., Roth, G.S. and Baum, B.J. Modification of ATP-dependent Ca^{2+} transport in rat parotid basolateral membranes during aging. Mech. Ageing Develop., 43: 45-60, 1988.

106.Ishikawa, Y., Gee, M.V., Ambudkar, I.S., Bodner, L., Baum, B.J. and Roth, G.S. Age-related impairment in rat parotid cell $alpha_1$-adrenergic action at the level of inositol trisphosphate responsiveness. Biochim. Biophys. Acta., 968: 203-210, 1988.

107.He, X., Wu, X. and Baum, B.J. Protein kinase C differentially inhibits muscarinic receptor operated Ca^{2+} release and entry in human salivary cells. Biochem. Biophys. Res. Commun. 152: 1062-69, 1988.

108.Fox, P.C., Wolff, A., Yeh, C.-K., Atkinson, J.C. and Baum, B.J. Saliva inhibits HIV-1 infectivity, J. Amer. Dent. Assoc. 116: 635-7, 1988.

109.Ambudkar, I.S. and Baum, B.J. Modulation of ATP-dependent Ca^{2+} transport in rat parotid basolateral membrane vesicles by $K^+ + Cl^-$ flux. Biochim. Biophys. Acta 941: 198-208, 1988.

110.Ambudkar, I.S. and Baum, B.J. ATP-dependent Ca^{2+} transport in rat parotid basolateral membrane vesicles is modulated by membrane potential. J. Membr. Biol. 102: 59-69, 1988.

111.Tylenda, C.A., Ship, J.A., Fox, P.C. and Baum, B.J. Evaluation of submandibular salivary flow rate in different age groups. J. Dent. Res. 67: 1225-1228, 1988.

112.Sonies, B.C., Parent, L.J., Morrish, K. and Baum, B.J. Durational aspects of the oral phase of swallow in normal aging adults. Dysphagia 3: 1-10, 1988.

113.Melvin, J.E., He, X. and Baum, B.J. Sympathetic denervation fails to produce β-adrenergic supersensitivity in adult rat parotid gland. J. Pharmacol. Exp. Thera. 246: 935-940, 1988.

114.Ambudkar, I.S., Melvin, J.E. and Baum, B.J. α_1-Adrenergic regulation of Cl^- and Ca^{2+} movements in rat parotid acinar cells. Pflugers Archiv. 412: 75-79, 1988.

115.Baum, B.J. and Ambudkar, I.S. Regulation of calcium handling by rat parotid acinar cells. Mol. Cell. Biochem. 82: 67-73, 1988.

116.Baum, B.J., Roberts, M.W., Brahim, J.S. and Fox, P.C. The National Institute of Dental Research Clinical Dental Staff Fellowship. J. Dent. Educ. 52: 535-536, 1988.

117.Baum, B.J., Ambudkar, I.S. and Horn, V.J. ATP-dependent Ca^{2+}-transport in rat parotid microsomal membranes requires charge compensation. Biochem. J. 254: 649-654, 1988.

118.Horn, V.J., Baum, B.J. and Ambudkar, I.S. β-Adrenergic receptor stimulation induces inositol trisphosphate production and Ca^{2+} mobilization in rat parotid acinar cells. J. Biol. Chem. 263: 12454-12460, 1988.

119.Wellner, R.B., He, X., Marmary, Y. and Baum, B.J. A human mammary cell line (HBL-100) possesses functional β-adrenergic receptors. Biochem. Pharmacol. 37: 3035-3037, 1988.

120.He, X., Wu, X., Brown, A.M., Wellner, R.B. and Baum, B.J. Characteristics of α_1-adrenergic receptors in a rat salivary cell line, RSMT-A5. Gen. Pharmacol. 20: 175-181, 1989.

121.Yeh, C.-K., Fox, P.C., Fox, C.H., Travis, W.D., Lane, H.C. and Baum, B.J. Kaposi's sarcoma of the parotid gland in acquired immunodeficiency syndrome (AIDS). Oral Surg. 67: 308-312, 1989.

122.Ishikawa, Y., Gee, M.V., Baum, B.J. and Roth, G.S. Characteristics of alpha$_1$-adrenergic receptors in intact and broken rat parotid cell preparations. Expt. Gerontol. 24: 25-36, 1989.

123.Tylenda, C.A. and Baum, B.J. Oral physiology and the Baltimore longitudinal study of aging. Gerodontology 7: 5-9, 1988.

124.Yeh, C.-K., Fox, P.C., Ship, J.A., Busch, K.A., Bermudez, D.K., Wilder, A-M, Katz, R.W., Wolff, A., Tylenda, C.A., Atkinson, J.C. and Baum, B.J. Oral defense mechanisms are impaired early in HIV-1 infected patients. J. Acquired Immune Deficiency Syndromes 1: 361-366, 1988.

125.Ambudkar, I.S., Horn, V.J. and Baum, B.J. ATP-dependent Ca^{2+} transport in the rat parotid basolateral membrane is regulated by calmodulin. Arch. Biochem. Biophys. 268: 576-584, 1989.

126.He, X., Wu, X., Wellner, R.B. and Baum, B.J. Muscarinic receptor regulation of Ca^{2+} mobilization in a human salivary cell line. Pflugers Archiv. 413: 505-510, 1989.

127.Brown, A.M., Rusnock, E.J., Sciubba, J.J. and Baum, B.J. Establishment and characterization of an epithelial cell line from the rat submandibular gland. J. Oral Path. 18: 206-213, 1989.

128.Brown, A.M. and Baum, B.J. System A amino acid transport in a rat submandibular ductal cell line. Comp. Biochem. Physiol. 93A: 383-389, 1989.

129.Mirels, L., Baum, B.J. and Kousvelari, E.E. Dissociation between c-fos gene expression and DNA synthesis in rat parotid glands. Arch. Oral Biol. 34: 511-515, 1989.

130.Wolff, A., Fox, P.C., Ship, J.A., Atkinson, J.C., Macynski, A.A. and Baum, B.J. Oral Mucosal status and major salivary gland function. Oral Surg. 70: 49-54, 1990.

131.Scott, J., Baum, B.J., Woods, K. and Berry, M.R. Chloride handling by submandibular cells from young and old rats with and without duct ligation. Mech. Ageing Develop. 48: 231-241, 1989.

132.Ship, J.A., DeCarli, C., Friedland, R.P. and Baum, B.J. Diminished submandibular salivary flow in dementia of the Alzheimer type. J. Gerontol. 45: M61-M66, 1990.

133.Sonies, B.C., Ship, J.A. and Baum, B.J. Relationship between saliva production and oropharygeal swallow in healthy, different-aged adults. Dysphagia. 4: 85-89, 1989.

134.Fox, P.C., Wolff, A., Yeh, C.-K., Atkinson, J.C. and Baum, B.J. Salivary inhibition of HIV-1 infectivity: functional properties and distribution in men, women and children. J. Amer. Dent. Assoc. 118: 709-711, 1989.

135.Atkinson, J.C., Yeh, C.-K., Oppenheim, F.G., Bermudez, D., Baum, B.J. and Fox, P.C. Elevation of salivary antimicrobial proteins following HIV-1 infection. J. Acquired Immune Deficiency Syndromes 3: 41-48, 1990.

136.Maki, T., Kowatch, M.A., Baum, B.J., Ambudkar, I.S. and Roth, G.S. Evidence for an alteration in the microsomal Ca^{2+} release mechanism in senescent rat parotid acinar cells. Biochim. Biophys. Acta. 1014: 73-77, 1989.

137.Weiffenbach, J.M., Tylenda, C.A. and Baum, B.J. Oral sensory changes in aging. J. Gerontol. 45: M121-M125, 1990.

138.Atkinson, J.C., Yeh, C.-K., Bermudez, D., Fox, P.C. and Baum, B.J. Longitudinal evaluation of major salivary gland function in HIV-1 infected patients. J. Oral Pathol. Baum, B.J., Yeh, C.-K. and Kousvelari, E.E. Influence of β-adrenergic stimulation on glycosylation of a major, secretory N-linked glycoprotein from rat parotid salivary gland. Arch. Oral Biol. 35: 201-207, 1990.

139.Wu, X., He, X. and Baum, B.J. Muscarinic regulation of Ca^{2+} mobilization in a human salivary myoepithelial cell line. Biochem. Pharmacol. 39: 612-615, 1990.

140.Horn, V.J., Baum, B.J. and Ambudkar, I.S. Evidence for the convergence of β-adrenergic and muscarinic signalling systems at a post-receptor site. FEBS Letters. 258: 13-16, 1989.

141.He, X., Wu, X. and Baum, B.J. The effect of N-(6 aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) on muscarinic receptor-induced Ca^{2+} mobilization in a human salivary epithelial cell line. Pflugers Archiv. 416: 36-42, 1990.

142.He, X., Wu, X., Turner, R.J. and Baum, B.J. Evidence for two modes of Ca^{2+} entry following muscarinic stimulation of a human salivary epithelial cell line. J. Membr. Biol. 115: 159-166, 1990.

143.Mertz, L.M., Horn, V.J., Baum, B.J. and Ambudkar, I.S. Calcium entry in rat parotid acini: activation by carbachol and aluminum fluoride. Amer. J. Physiol.: Cell Physiology. 258: C654-C661, 1990.

144.Horn, V.J., Baum, B.J. and Ambudkar, I.S. Attenuation of inositol trisphosphate generation and cytosolic Ca^{2+} elevation in dispersed rat parotid acini stimulated simultaneously at muscarinic and (1-adrenergic receptors. Biochem. Biophys. Res. Commun. 166: 967-972, 1990.

145.Wolff, A., Ship, J.A., Tylenda, C.A., Fox, P.C. and Baum, B.J. Oral mucosal appearance is unchanged in healthy, different-aged individuals. Oral Surg. 71: 569-572, 1991.

146.Mertz, L.M., Baum, B.J. and Ambudkar, I.S. Refill status of the agonist-sensitive Ca^{2+} pool regulates Mn^{2+} influx into parotid acini. J. Biol. Chem. 265: 15010-15014, 1990.

147.Ambudkar, I.S., Horn, V.J., Dai, Y. and Baum, B.J. Evidence against a role for a pertussis toxin-sensitive G-protein in Ca^{2+} mobilization in rat parotid acinar cells. Biochim. Biophys. Acta. 1055: 259-264, 1990.

148.Ship, J.A., Fox, P.C. and Baum, B.J. How much saliva is enough? J. Amer. Dent. Assoc. 122: 63-69, 1991.

149.Ship, J.A. and Baum, B.J. Is reduced salivary flow normal in old people? (letter) Lancet. 336: 1507, 1990.

150.Hiramatsu, Y., Ambudkar, I.S. and Baum, B.J. Characterization of high affinity GTPase activity correlated to β-adrenergic receptor stimulation of adenylyl cyclase in rat parotid membranes. Biochim. Biophys. Acta. 1092: 391-396, 1991.

151.Fox, P.C., Atkinson, J.C., Macynski, A.A., Wolff, A., Kung, D.S., Valdez, I.H., Jackson, W., Delapenha, R.A., Shiroky, J. and Baum, B.J. Pilocarpine treatment of salivary gland hypofunction and dry mouth (xerostomia). Arch. Int. Med. 151: 1149-1152, 1991.

152.Baum, B.J. Has modern biology entered the mouth? The clinical impact of biological research. J. Dent. Educ. 55: 299-303, 1991.

153.Royce, L.S. and Baum, B.J. Physiologic levels of salivary epidermal growth factor stimulate migration of an oral epithelial cell line. Biochim. Biophys. Acta. 1092: 401-403, 1991.

154.Yeh, C.-K., Mertz, P.M., Oliver, C., Baum, B.J. and Kousvelari, E.E. Cellular characteristics of long term cultured rat parotid acinar cells. In Vitro Cell. Develop. Biol. 27A:707-712, 1991.

155.Horn, V.J., Ambudkar, I.S. and Baum, B.J. High affinity quinuclidinyl benzilate binding to rat parotid membranes requires muscarinic receptor: G protein interactions. FEBS Lett. 282: 289-292, 1991.

156.Horn, V.J., Baum, B.J. and Ambudkar, I.S. Muscarinic receptor antagonist effects in parotid acini and M_1-CHO cells: evidence of G protein involvement. Biochem. Biophys. Res. Commun. 177: 784-789, 1991.

157.Dai, Y., Ambudkar, I.S., Horn, V.J., Yeh, C.-K., Kousvelari, E.E., Wall, S.J., Li, M., Yasuda, R.P., Wolfe, B.B. and Baum, B.J. Evidence that M_3 muscarinic receptors in rat parotid gland couple to two second messenger systems. Amer. J. Physiol. 261:C1063-C1073, 1991.

158.Cherry-Peppers, G., Sorkin, J., Andres, R., Baum, B.J. and Ship, J.A. Salivary gland function and glucose metabolic status. J. Gerontol. 47:M130-M134, 1992.

159.Mertz, L.M., Baum, B.J. and Ambudkar, I.S. Membrane potential modulates divalent cation entry in rat parotid acini. J. Membr. Biol. 126:183-193, 1992.

160.Kohn, W.G., Grossman, E., Fox, P.C., Armando, I., Goldstein, D.S. and Baum, B.J. Effect of ionizing radiation on sympathetic nerve function in rat parotid gland. J. Oral Path. Med. 21:134-137, 1992.

161.Mertz, P.M., Fox, P.C., Pluta, A., Baum, B.J. and Kousvelari, E.E. Effects of ionizing radiation and β-adrenergic stimulation on the expression of early response genes in rat parotid glands. Radiat. Res. 130:104-112, 1992.

162.Kibbey, M.C., Royce, L.S., Dym, M., Baum, B.J. and Kleinman, H.K. Glandular-like morphogenesis of the human submandibular tumor cell line A253 on basement membrane components. Exp. Cell Res. 198:343-351, 1992.

163.Yeh, C.-K., Handelman, B., Fox, P.C. and Baum, B.J. Further studies of salivary inhibition of HIV-1 infectivity. J. Acquired Immune Deficiency Syndromes 5:898-903, 1992.

164.Hiramatsu, Y., Baum, B.J. and Ambudkar, I.S. Elevation of cytosolic [Ca^{2+}] due to intracellular Ca^{2+} release retards carbachol stimulation of divalent cation entry in rat parotid gland acinar cells. J. Membr. Biol. 129:277-286, 1992.

165.Baum, B.J., Ship, J.A. and Wu, A.J. Salivary gland function and aging: a model for studying the interaction of aging and systemic disease. Crit. Rev. Oral Biol. Med. 4:53-64, 1992.

166.Hiramatsu, Y., Horn, V.J., Baum, B.J. and Ambudkar, I.S. Characterization of polyphosphoinositide-specific phospholipase C in rat parotid membranes. Arch. Biochem. Biophys. 297:368-376, 1992.

167.Baum, B.J., Dai, Y., Hiramatsu, Y., Horn, V.J. and Ambudkar, I.S. Signaling mechanisms which regulate saliva formation. Crit. Rev. Oral Biol. Med. 4:379-384, 1993.

168.Ambudkar, I.S., Hiramatsu, Y., Lockwich, T. and Baum, B.J. Activation and regulation of Ca^{2+} influx in rat parotid acinar cells. Crit. Rev. Oral Biol. Med. 4:421-425, 1993.

169.Hiramatsu, Y., Kawai, R., Reba, R.C., Simon, T.R., Baum, B.J. and Blasberg, R.G. Kinetic analysis of rat parotid gland muscarinic receptors in vivo: comparison with brain and heart. Am. J. Physiol. 264:G541-G552, 1993.

170.Royce, L.S., Kibbey, M.C., Mertz, P., Kleinman, H.K. and Baum, B.J. Human neoplastic submandibular intercalated duct cells express an acinar phenotype when cultured on a basement membrane matrix. Differentiation 52:247-255, 1993.

171.Ship, J.A. and Baum, B.J. Old age in health and disease: lessons from the oral cavity. Oral Surg. 76:40-44, 1993.

172.Wu, A.J., Atkinson, J.A., Fox, P.C., Baum, B.J. and Ship, J.A. Cross-sectional and longitudinal analyses of stimulated parotid salivary constituents in healthy, different aged subjects. J. Gerontol. 48:M219-M224, 1993.

173.Nagler, R.M., Baum, B.J. and Fox, P.C. Acute effects of x-irradiation on the function of rat salivary glands. Radiat. Res. 136:42-47, 1993.

174.Sawaki, K., Hiramatsu, Y., Baum, B.J. and Ambudkar, I.S. Involvement of $G_{aq/11}$ in m_3-muscarinic receptor stimulation of phosphatidylinositol 4, 5 bisphosphate-specific phospholipase C in rat parotid gland membranes. Arch. Biochem. Biophys. 305:546-550, 1993.

175.Ambudkar, I.S., Lockwich, T., Hiramatsu, Y. and Baum, B.J. Calcium entry in rat parotid acinar cells. Mol. Cell. Biochem. 114:73-77, 1992.

176.Dai, Y. and Baum, B.J. Relationship between muscarinic receptor occupancy and response in rat parotid acinar cells. Am. J. Physiol. 265:G1122-G1127, 1993.

177.Nagler, R.M., Baum, B.J. and Fox, P.C. Effects of x-irradiation on the function of rat salivary glands at 3 and 40 days. Radiat. Res. 136:392-396,1993.

178.Mastrangeli, A., O'Connell, B., Aladib, W., Fox, P.C., Baum, B.J. and Crystal, R.G. Direct in vivo adenovirus-mediated gene transfer to salivary glands. Am. J. Physiol. 266:G1146-G1155, 1994.

179.Hiramatsu, Y., Nagler, R.M., Fox, P.C. and Baum, B.J. Rat salivary gland blood flow and blood-to-tissue partition coefficients following x-irradiation. Arch. Oral Biol. 39:77-80,1994.

180.Wu, A.J., Kurrasch, R.H., Katz, J., Fox, P.C., Baum, B.J. and Atkinson, J.C. The effect of tumor-necrosis factor-α and interferon-γ on the growth of a human salivary gland cell line. J. Cell. Physiol. 161:217-226,1994.

181.Hiramatsu, Y., Kawai, R., Reba, R.C., Blasberg, R.G. and Baum, B.J. Kinetic analysis of rat exocrine gland muscarinic receptors in vivo. J. Pharmacol. Expt. Thera. 269:1205-1212,1994.

182.Hiramatsu, Y., Eckelman, W.C. and Baum, B.J. Interaction of iodinated quinuclidinyl benzilate enantiomers with M3 muscarinic receptors. Life Sciences 54:1777-1783, 1994.

183.Wu, A.J., Baum, B.J. and Ship, J.A. Extended stimulated parotid and submandibular secretion in a healthy, young and old population. J. Gerontol. 50A:M45-M48, 1995.

184.Li, J., Nielsen, S., Dai, Y., Lazowski, K.W., Christensen, E.I., Tabak, L.A. and Baum, B.J. Examination of rat salivary glands for the presence of the aquaporin CHIP. Pflugers Archiv. 428:455-460,1994.

185.Katz, J., Nagler, R., Barak, S., Livneh, A., Baum, B., Atkinson, J. and Shemer, J. Cytokines modulate interleukin-6 production by a human salivary gland cell line. Cellular Immunol. 159:211-219,1994.

186.Sawaki, K., Baum, B.J. and Ambudkar, I.S. α_1-Adrenergic and m_3-muscarinic receptor stimulation of phosphatidylinositol 4,5-bisphosphate-specific phospholipase C are independently mediated by $G_{aq/11}$ in rat parotid gland membranes. Arch. Biochem. Biophys. 316:535-540,1995.

187.Hiramatsu, Y., Eckelman, W.C., Carrasquillo, J.A., Miletich, R.S., Valdez, I.H., Kurrasch, R.H.M., Macynski, A.A., Paik, C.H., Neumann, R.D. and Baum, B.J. Kinetic analysis of muscarinic receptors in human brain and salivary gland in vivo. Am. J. Physiol. 268:R1491-R1499,1995.

188.Lazowski, K.W., Li, J., Delporte, C. and Baum, B.J. Evidence for the presence of a Hg-inhibitable water-permeability pathway and aquaporin 1 in A5 salivary epithelial cells. J. Cell. Physiol. 164:613-619, 1995.

189.O'Connell, B.C. Ten Hagen, K.G., Lazowski, K.W., Tabak, L.A. and Baum, B.J. Facilitated DNA transfer to rat submandibular gland in vivo and GRP-Ca gene regulation. Am. J. Physiol. 268:G1074-G1078,1995.

190.Sawaki, K., Baum, B.J., Roth, G.S. and Ambudkar, I.S. Decreased m_3-muscarinic and α_1-adrenergic receptor stimulation of PIP_2 hydrolysis in parotid gland membranes from aged rats: defect in activation of $G_{aq/11}$. Arch. Biochem. Biophys. 322:319-326, 1995.

191.Nagler, R.M., Baum, B.J. and Fox, P.C. Early x-irradiation effects on rat major salivary gland function: a 2 week pair-fed study. Archs. Oral Biol. 41:713-717, 1996.

192.Wu, A.J., Chen, Z.J., Baum, B.J. and Ambudkar, I.S. Interferon-γ induces persistent depletion of intracellular Ca^{2+} stores in a human salivary gland cell line. Am. J. Physiol. 270:C514-C521, 1996.

193.Wu, A.J., Chen, Z.J., Tsokos, M., O'Connell, B.C., Ambudkar, I.S. and Baum, B.J. Interferon-γ induced cell death in a cultured human salivary gland cell line. J. Cell. Physiol. 167:297-304, 1996.

194.Adesanya, M., Redman, R.S., Baum, B.J. and O'Connell, B.C. Immediate inflammatory responses to adenovirus-mediated gene transfer in rat salivary glands. Hum. Gene Ther. 7:1085-1093, 1996.

195.Kagami, H., O'Connell, B.C., and Baum, B.J. Evidence for the systemic delivery of a transgene product from salivary glands. Hum. Gene Ther. 7:2177-2184, 1996.

196.He, X., Tse, C.-M., Donowitz, M., Alper, S.L., Gabriel, S.E., and Baum, B.J. polarized distribution of key membrane transport proteins in the rat submandibular gland. Pflugers Archiv. 433:260-268, 1997.

197.Delporte, C., O'Connell, B.C., He, X., Ambudkar, I.S., Agre, P. and Baum, B.J.Adenovirus-mediated expression of aquaporin-5 in epithelial cells. J. Biol. Chem. 271:22070-22075, 1996.

198.O'Connell, B.C., Xu, T., Walsh, T.J., Sein, T., Mastrangeli, A., Crystal, R.G,Oppenheim, F.G. and Baum, B.J. Transfer of a gene encoding the anticandidal protein histatin 3 to salivary glands. Hum. Gene Ther. 7:2255-2261, 1996.

199.Nagler, R., Marmary, Y., Fox, P.C., Baum, B.J., Har-El, R. and Chevion, M. Irradiation-induced damage to the salivary glands: the role of redox-active iron and copper. Radiat. Res. 147:468-475, 1997.

200.Delporte, C., Chen, Z.J. and Baum, B.J. Aquaporin 1 expression during the cell cycle in A5 cells. Biochem. Biophys. Res. Commun. 228:223-228, 1996.

201.Wu, A.J., Lafrenie, R.M., Park, C., Apinhasmit, W., Chen, Z.J., Birkedal-Hansen, H., Yamada, K.M., Stetler-Stevenson, W.G. and Baum, B.J. Modulation of MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) by interferon-γ in a human salivary gland cell line. J. Cell. Physiol. 171:117-124, 1997.

202.Delporte, C., O'Connell, B.C., He, X., Lancaster, H.E., O'Connell, A.C., Agre, P. and Baum, B.J. Increased fluid secretion following adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. Proc. Natl. Acad. Sci. USA 94:3268-3273, 1997.

203.Wu, A.J., Chen, Z.J., Kan, E.C. and Baum, B.J. Interferon-γ induced JAK2 and STAT1 signalling in a human salivary gland cell line. J. Cell. Physiol. 173:110-114, 1997.

204.Delporte, C., Miller, G., Kagami, H., Lillibridge, C.D., O'Connell, B.C., Atkinson, J.C. and Baum, B.J. Safety of salivary gland-administered replication-deficient recombinant adenovirus in rats. J. Oral Pathol. Med. 27:34-38, 1998.

205.Delporte, C., Redman, R.S. and Baum, B.J. Relationship between the cellular distribution of the $\alpha_v\beta_{3/5}$ integrins and adenoviral infection in salivary glands. Lab. Invest. 77:167-173, 1997.

206.Delporte, C., Panyutin, I.G., Sedelnikova, O.A., Lillibridge, C.D., O'Connell, B.C. and Baum, B.J. Triplex forming oligonucleotides can modulate aquaporin-5 gene

expression in epithelial cells. Antisense & Nucleic Acid Drug Develop. 7:523-529, 1997.

207.Nagler, R., Baum, B.J., Miller, G. and Fox, P.C. Long-term effects of single dose regional head and neck irradiation in the rat. Arch. Oral Biol. 43:297-303, 1998.

208.He, X., Kuijpers G.A.J., Goping, G., Kulakusky, J.A., Zheng, C., Delporte, C., Tse, C.-M., Redman, R.S., Donowitz, M., Pollard, H.B. and Baum, B.J. A polarized salivary cell monolayer useful for studying transepithelial fluid movement in vitro. Pflugers Archiv 435:375-381, 1998.

209.Kagami, H., Atkinson, J.C., Michalek, S.M., Handelman, B., Yu, S., Baum, B.J. and O'Connell, B. Repetitive adenovirus administration to the parotid gland: role of immunological barriers and induction of oral tolerance. Human Gene Ther. 9:305-313, 1998.

210.He, X., Goldsmith, C.M., Marmary, Y., Wellner, R.B., Parlow, A.F., Nieman, L.K. and Baum, B.J. Systemic action of human growth hormone following adenovirus-mediated gene transfer to rat submandibular glands. Gene Ther. 5:537-541, 1998.

211.O'Connell, A.C., Lillibridge, C.D., Zheng, C., Baum, B.J., O'Connell, B.C. and Ambudkar, I.S. γ-Irradiation-induced cell cycle arrest and cell death in a human submandibular gland cell line: effect of E2F1 expression. J. Cell. Physiol. 177: 264-273.

212.Delporte, C., Hoque, A.T.M.S., Kulakusky, J.A., Braddon, V.R., Goldsmith, C.M., Wellner, R.B. and Baum, B.J. Relationship between adenovirus-mediated aquaporin 1 expression and fluid movement across epithelial cells. Biochem. Biophys. Res. Commun. 246:584-588, 1998.

213.Baum, B.J. and Davis, C. Clinical research, dental education, and the NIH clinical research training program. J. Dental Educ. 62:592-596, 1998.

214.Braddon, V.R., Chiorini, J.A., Wang, S., Kotin, R.M. and Baum, B.J. Adenoassociated virus-mediated transfer of a functional water channel into salivary epithelial cells in vitro and in vivo. Human Gene Ther. 9:2777-2785, 1998.

215.Marmary, Y., Parlow, A.F., Goldsmith, C.M., He, X., Wellner, R.B., Satomura, K., Kriete, M.F., Robey, P.G., Nieman, L.K. and Baum B.J. Construction and in vivo efficacy of a replication-deficient recombinant adenovirus encoding murine growth hormone. Endocrinology 140:260-265, 1999.

216.Wang, S., Cukierman, E., Swaim, W.D., Yamada, K.M. and Baum, B.J. Extracellular matrix protein-induced changes in human salivary epithelial cell organization and proliferation on a model biological substratum. Biomaterials 20:1043-1049, 1999.

217.O'Connell, A.C., Baccaglini, L., Fox, P.C., O'Connell, B.C., Kenshalo, D., Oweisy, H., Hoque, A.T.M.S., Sun, D., Herscher, L.L., Braddon, V.R., Delporte, C. and Baum, B.J. Safety and efficacy of adenovirus-mediated transfer of the human aquaporin 1 cDNA to irradiated parotid glands of non-human primates. Cancer Gene Ther. 6:505-513, 1999.

218.Wang, S., Baum, B.J., Kagami, H., Zheng, C., O'Connell, B.C., and Atkinson, J.C. Effect of clodronate on macrophage depletion and adenoviral-mediated transgene expression in salivary glands. J. Oral Pathol. Med. 28:145-151, 1999.

219.Yamano, S., Atkinson, J.C., Baum, B.J., and Fox, P.C. Salivary gland cytokine expression in NOD and normal BALB/C mice. Clin. Immunol. 92:265-275, 1999.

220.Baum, B.J., Berkman, M.E., Marmary, Y., Goldsmith, C.M., Baccaglini, L., Wang, S., Wellner, R.B., Hoque, A.T.M.S., Atkinson, J.C., Yamagishi, H., Kagami, H., Parlow, A.F. and Chao, J. Polarized secretion of transgene products from salivary glands in vivo. Human Gene Ther. 10: 2789-2797, 1999.

221.Ghezzi, E.M., Wagner-Lange, L.A., Schork, A., Metter, J. Baum, B.J., Streckfus, C.F., and Ship, J.A. Longitudinal influence of age, menopause, hormone replacement therapy, and other medications on parotid flow rates in healthy women. J. Gerontol. 55: M34-M42, 2000.

222.Hoque, A.T.M.S., Liu, X., Kagami, H. Swaim, W.D., Wellner, R.B., O'Connell, B.C., Ambudkar, I.S., and Baum, B.J. Construction and function of a recombinant adenovirus encoding a human aquaporin 1-green fluorescence protein fusion product. Cancer Gene Ther. 7: 476-485, 2000.

223.Wang, S., Baum, B.J., Yamano, S., Mankani, M.H., Sun, D., Jonsson, M., Davis, C., Graham, F.L., Gauldie, J. and Atkinson, J.C. Adenoviral-mediated gene transfer to mouse salivary glands. J. Dent. Res. 79: 701-708, 2000.

224. Aframian, D., Cukierman, E., Nikolovski, J., Mooney, D.J., Yamada, K.M. and Baum, B.J. The growth and morphological behavior of salivary epithelial cells on matrix protein-coated biodegradable substrata. Tissue Engineering 6: 209-216, 2000.

225.Zheng, C., Baum, B.J., Iadarola, M. J. and O'Connell, B.C. Genomic integration and long term expression by a modified adenoviral vector. Nature Biotechnol. 18: 176-180, 2000.

226.Baccaglini, L., Pillemer, S.R. and Baum, B.J. Sjogren's syndrome: a possible pathogenic mechanism involving somatostatin. Oral Diseases 6: 264-266, 2000.

227.Hoque, A.T.M.S., Sedelnikova, O.A., Luu, A.N., Swaim, W.D., Panyutin, I.G. and Baum, B.J. Use of polyethylenimine-adenovirus complexes to examine triplex

formation in intact cells. Antisense & Nucleic Acid Drug Develop. 10: 229-241, 2000.

228.Wellner, R.B., Hoque, A.T.M.S., Goldsmith, C.G. and Baum, B.J. Evidence for a basolateral localization of aquaporin-8 in rat submandibular gland acinar cells. Pflugers Archiv 441: 49-56, 2000.

229.Zheng, C., Goldsmith, C.M., O'Connell, B.C. and Baum, B.J. Adenoviral vector cytotoxicity depends in part on the transgene encoded. Biochem. Biophys. Res. Commun. 274:767-771, 2000.

230.Slavkin, H.C. and Baum, B.J. The relation of dental and oral diseases to systemic illness. JAMA 284: 1215-1217, 2000.

231.Baccaglini, L., Hoque, A.T.M.S., Wellner, R.B., Goldsmith, C.M., Redman, R.S., Sankar, V., Kingman, A., Barnhart, K.M., Wheeler, C.J. and Baum, B.J. Cationic liposome-mediated gene transfer to rat salivary epithelial cells in vitro and in vivo. J. Gene Med. 3:82-90, 2001.

232.Aframian, D.J., Zheng, C., Goldsmith, C.M., Nikolovski, J., Cukierman, E., Yamada, K.M., Mooney, D.J., Birkedal-Hansen, H. and Baum, B.J. Using HSV-thymidine kinase for safety in an allogeneic salivary graft cell line. Tissue Engineering 7:405-413, 2001.

233.Heron, L., Karas, M., Goldsmith, C.M., Baum, B.J. and LeRoith, D. IGF-1 receptor activation rescues UV-damaged cells through a p38 signaling pathway: implications for the role of the IGF-1 receptor in DNA repair. J. Biol. Chem. 276:18185-18192, 2001.

234.Wellner, R.B. and Baum, B.J. Polarized sorting of aquaporins 5 and 8 in stable MDCK-II transfectants. Biochem. Biophys. Res. Commun. 285: 1253-1258, 2001.

235.Hoque, A.T.M.S., Baccaglini, L. and Baum, B.J. Hydroxychloroquine enhances the endocrine secretion of adenovirus-directed growth hormone from rat submandibular glands in vivo. Human Gene Ther. 12:1333-1341, 2001.

236.Yamano, S., Scott, D.E., Huang, L.Y., Mikolajczyk, M., Pillemer, S.R., Chiorini, J.A., Golding, B. and Baum, B.J. Protection from experimental endotoxemia by a recombinant adeno-associated virus encoding interleukin 10. J. Gene Med. 3:450-457, 2001.

237.Aframian, D.J., Redman, R.S., Yamano, S., Nikolovski, J., Cukierman, E., Yamada, K.M., Kriete, M.F., Swain, W.D., Mooney, D.J. and Baum, B.J. Tissue compatability of two biodegradable tubular scaffolds implanted adjacent to skin or buccal mucosa in mice. Tissue Engineering 8:649-659, 2002.

238.Zheng, C. Hoque, A.T.M.S., Braddon, V.R., Baum, B.J. and O'Connell, B.C. Evaluation of salivary gland acinar and ductal cell-specific promoters in vivo with recombinant adenoviral vectors. Human Gene Ther. 12:2215-2223, 2001.

239.Aframian, D.J., Tran, S.D., Cukierman, E., Yamada, K.M. and Baum, B.J. Absence of tight junction formation in an allogeneic graft cell line used for developing an engineered artificial salivary gland. Tissue Engineering 8:871-878, 2002.

240.Yamano, S. Huang, L.Y., Ding, C., Chiorini, J.A., Goldsmith, C.M., Wellner, R.B., Golding, B., Kotin, R.M., Scott, D.E. and Baum, B.J. Recombinant adeno-associated virus serotype 2 vectors mediate stable interleukin 10 secretion from salivary glands into the bloodstream. Human Gene Ther. 13:287-298, 2002.

241.Zheng, C. and Baum, B.J. Long-term expression after infection by the hybrid vector AdLTR-luc is from integrated transgene. Biochem. Biophys. Res. Commun. 291:34-40, 2002.

242.Brennan, M.T., Sankar, V., Leakan, R.A., Kleiner, D., Atkinson, J.C., Wilkinson, W.E., Baum, B.J. and Pillemer, S.R. Risk factors for positive minor salivary gland biopsy findings in Sjogren's syndrome and dry mouth patients. Arthritis Care & Res. 47:189-195, 2002.

243.Hoque, A.T.M.S., Yamano, S., Liu, X., Swaim, X., Goldsmith, C.M., Delporte, C. and Baum, B.J. Expression of the aquaporin 8 water channel in a rat salivary epithelial cell line. J. Cell. Physiol. 191:336-341, 2002.

244.Radfar, L., Shea, Y., Sankar, V., Leakan, R.A., Baum, B.J., Fischer, S.H. and Pillemer, S.R. Fungal load and candidiasis in Sjogren's syndrome. Oral Surg. 96:283-287, 2003.

245.Zheng, C. Wang, J. and Baum, B.J. Integration efficiency of a hybrid adenoretroviral vector. Biochem. Biophys. Res. Commun. 300: 115-120, 2003.

246.Tran, S.D., Pillemer, S.R., Dutra, A., Barrett, A.J., Brownstein, M.J., Key, S., Pak, E., Leakan, R.A., Kingman, A., Yamada, K.M., Baum, B.J. and Mezey, E. Differentiation of human bone marrow-derived cells into buccal epithelial cells in vivo: a molecular analytical study. Lancet 361:1084-1088, 2003.

247.O'Connell, B. C., Zheng, C., Jacobson-Kram, D. and Baum, B.J. Distribution and toxicity resulting from adenoviral vector administration to a single salivary gland in adult rats. J. Oral Pathol. Med. 32:414-421, 2003.

248. Zheng, C., O'Connell, B. C. and Baum, B.J. Inclusion of Moloney murine leukemia virus elements upstream of the transgene cassette in an E1-deleted adenovirus leads to an unusual genomic integration in epithelial cells. Virology 313:460-472, 2003.

249. Kok, M.R., Baum, B.J., Tak, P.P. and Pillemer, S.R. Use of localized gene transfer to develop new treatment strategies for the salivary component of Sjogren's syndrome. Ann. Rheum. Dis. 62: 1038-1046, 2003.

250. Kok, M.R., Yamano, S., Lodde, B.M., Wang, J., Couwenhoven, R.I., Yakar, S., Voutetakis, A., Leroith, D., Schmidt, M., Afione, S., Pillemer, S.R., Tsutsui, M.T., Tak, P.P., Chiorini, J.A. and Baum, B.J. Local adeno-associated virus-mediated interleukin-10 gene transfer has disease modifying effects in a murine model of Sjogren's syndrome. Human Gene Ther. 14: 1605-1618, 2003.

251. Aframian, D., David, R., Ben-Basset, H., Shai, E., Deutsch, D., Baum, B.J. and Palmon, A. Characterization of murine salivary gland graft cells: a model for use with an artificial salivary gland. Tissue Engineering (in press).

252. Li, J., Zheng, C., Zhang, X., Liu, X., Zhang, C., Goldsmith, C.M., Baum, B.J. and Wang, S. Developing a convenient large animal model for gene transfer to salivary glands in vivo. J. Gene Med. (in press).

253. Wang, J., Voutetakis, A., Zheng, C. and Baum, B.J. Rapamycin control of exocrine protein levels in saliva after adenoviral-mediated gene transfer. Gene Ther. (in press).

Invited publications and reviews:

I1. Cohen, M.M., Baum, B.J., Garn, S.M., Nagy, H.M. and Osorio, C.H.: Crown size reduction in congential defects. In: Dahlberg, A.A. and Graber, T.M. (Eds.): Orofacial Growth and Development, The Hague, Mouton and Co., 1977, pp. 119-126.

I2. Baum, B.J., Bird, J.L., Berzinskas, V.J. and Longton, R.W.: Synthesis of histidine-rich-polypeptides by monkey parotid glands. In: Kleinberg, I. (Ed.): Saliva and Dental Caries, New York, Information Retrieval Inc., 1979, pp. 89-97.

I3. Baum, B.J.: Aging and oral motor function. In: Bryant, P., Gale, E. and Rugh, J. (Eds.): Oral Motor Behavior: Impact on Oral Conditions and Dental Treatment. NIH Publication No. 79-1845, Bethesda, 1979, pp. 244-252.

I4. Baum, B.J.: Salivary glands and saliva: recent advances in understanding their function and role in oral health. The New Dentist 9: 21-26, 1979.

I5. Baum, B.J.: Research on aging and oral health: An assessment of current status and future needs. NIA Report for 1981 White House Conference on Aging, Special Care in Dentistry 1: 156-165, 1981.

I6. Baum, B.J., Ito, H. and Roth, G.S.: Adrenoreceptors and the regulation of salivary gland physiology. In: Kunos, G. (Ed.): Adrenoreceptors and Catecholamine Action, Part B, 265-294.

I7. Baum, B.J.: Normal and Abnormal Oral Status in Aging. Ann. Rev. Geriatrics Gerontology, 4: 87-105, 1984.

I8. Baum, B.J.: Alterations in Oral Function. In: Principles of Geriatric Medicine (R. Andres et al., eds.), 288-295, 1985.

I9. Bienkowski, R.S. and Baum, B.J.: Measurement of intracellular protein degradation. In: Adelman, R. and Roth, G. (Eds.): Methods in Aging Research. Detection of Altered Proteins. CRC Press, Boca Raton, FL. pp. 55-80, 1983.

I10. Baum, B.J.: Age changes in salivary glands and salivary secretion. In: Holm-Pedersen, P. and Loe, H. (Eds.). Geriatric Dentistry. pp. 114-122, 1986.

I11. Baum, B.J.: The dentistry-gerontology connection. J. Amer. Dent. Assoc. 899-900, 1984.

I12. Somerman, M.J., Hoffeld, J.T. and Baum, B.J. Basic biology and physiology of oral tissues: overview and age-associated changes, In Oral Health and Aging (A. Tryon, Ed.) PSG Publishing, Littleton, MA, pp. 23-45, 1986.

I13. Baum, B.J. Saliva secretion and composition. In: Frontiers in Oral Physiology-The Aging Mouth. Ferguson, D.B. (ed), vol. 6, pp. 126-134, Karger, Basel, 1987.

I14. Baum, B.J. and Fox, P.C. Chemistry of saliva. In: Sjogren's Syndrome: Chemical and Immunological Aspects, Talal, N. et al. (eds)., pp. 34-40, Springer-Verlag, Berlin, 1987.

I15. Baum, B.J. Regulation of salivary secretion. In: The Salivary System., Sreebny. L.M. (ed)., pp. 123-134, Boca Raton, CRC Press, 1987.

I16. Baum, B.J. Salivary gland function during aging. Gerodontics 2: 61-64, 1986.

I17. Baum, B.J. Neurotransmitter control of secretion. J. Dent. Res. 66: 628-632, 1987.

I18. Weiffenbach, J.M., Fox, P.C. and Baum, B.J. Taste and salivary gland dysfunction. In: Olfaction and Taste IX. (S. Roper and J. Atema, Eds), New York Acad. Sci., pp. 698-699, 1987.

I19. Baum, B.J. The oral cavity. In: Geriatric Medicine, Rowe, J. and Besdine, R. (eds), pp. 157-166, Boston, Little-Brown, 1988.

I20. Sonies, B.C. and Baum, B.J. Evaluation of swallowing pathophysiology. Otolaryngol. Clin. North America 21: 637-648, 1988.

I21. Baum, B.J., Burstone, C.J., Dubner, R., Goldhaber, P., Levine, M.J., Loesche, W.J. and Terranova, V. Advances in diagnosis and detection of oral disease. Adv. Dent. Res. 3: 7-13, 1989.

I22. Baum, B.J., Ambudkar, I.S., Helman, J., Horn, V.J., Melvin, J.E., Mertz, L.M. and Turner, R.J. Dispersed salivary gland acinar cell preparations for use in studies of neuroreceptor coupled secretory events. Methods Enzymol. 192: 26-37, 1990.

I23. Scott, J. and Baum, B.J. Oral effects of ageing. In: Jones, H. and Mason, D. (eds). Oral manifestations of systemic disease. Bailliere Tindall, London, pp. 311-338, 1990.

I24. Baum, B.J. Salivary gland fluid secretion during aging. J. Amer. Geriatrics Soc. 37: 453-458, 1989.

I25. Baum, B.J. Dental and oral disorders. In: Abrams, W.B. and Berkow, R. (eds). The Merck Manual of Geriatrics, pp. 466-475, 1990.

I26. Baum, B.J. and Ship, J.A. The oral cavity. In: Hazzard, W.R., Andres, R., Bierman, E.L., and Blass, J.P. Principles of Geriatric Medicine and Gerontology,

pp. 413-422, 1990.

I27. Baum, B.J. Diseases of salivary glands and sensory impairment: state of the art. In: Millard, H.D. and Mason, D.K. (eds). World Workshop on Oral Medicine, Yearbook Medical Publishers, Chicago, IL, pp. 285-288, 1989.

I28. Baum, B.J., Caruso, A.J., Ship, J.A. and Wolff, A. Oral physiology. In: Papas, T., Niessen, L. and Chauncey, H. (eds). Geriatric Dentistry. pp. 71-82, 1991.

I29. Baum, B.J., Ambudkar, I.S. and Horn, V.J. Neurotransmitter control of calcium mobilization. In: Dobrosielski-Vergona, K. (ed). The Biology of Salivary Glands, CRC Press, Boca Raton, FL, pp. 153-179,1993.

I30. Baum, B.J. and Ship, J.A. Oral Disorders. In: Beck, J. (ed). American Geriatrics Society Geriatrics Review Syllabus, pp. 332-336, 1991.

I31. Baum, B.J. and Ambudkar, I.S. Regulation of calcium mobilization in rat parotid acinar cells. In: Wong, P.Y.D. and Young, J.A. (eds). Exocrine Secretion II. University of Hong Kong Press, pp. 9-12, 1990.

I32. Ambudkar, I.S., Lockwich, T., Hiramatsu, Y. and Baum, B.J. Regulation of calcium entry in rat parotid acinar cells: possible role of membrane potential and cytosolic Ca2+. In: Biotechnology of Cell Regulation, Verna, R. and Nishizuka, Y. (eds), Raven Press, New York, pp. 35-42.

I33. Baum, B.J. Age changes in salivary glands and salivary secretion. In: Holm-Pedersen, P. and Loe, H. (eds). Geriatric Dentistry, 2nd edition, Munksgaard, Copenhagen , pp. 117-126, 1996.

I34. Baum, B.J. and Fox, P.C. Commentary: recovery of infectious HIV-1 from whole saliva. J. Amer. Dent. Assoc. 123:38, 1992.

I35. Baum, B.J. Age-related vulnerability. Otolaryngol. Head Neck Surg. 106:730-732, 1992.

I36. Ship, J.A. and Baum, B.J. Editorial: Oral health and function in the elderly. Rev. Clin. Gerontol. 3:205-206, 1993.

I37. Baum, B.J. Commentary: education for dental practice in the year 2005: a beginning dialogue. J. Dent. Educ. 56:377-378, 1992.

I38. Baum, B.J. and Ship, J.A. The oral cavity. In: Hazzard, W.R., Bierman, E.L., Blass, J.P., Ettinger, W.H. and Halter, J.B. (eds). Principles of Geriatric Medicine and Gerontology, 3rd edition, McGraw-Hill, New York, pp. 431-439, 1994.

I39. Baum, B.J., Hiramatsu, Y., Kohn, W.G. and Valdez, I.H. New approaches for

determining the site of salivary fluid secretory disorders. Adv. Dent. Res. 7:220-224, 1993.

I40. Baum, B.J. Principles of salivary secretion. In: Malamud, D. and Tabak, L.A. (eds). Saliva as a diagnostic fluid. Ann. N.Y. Acad. Sci. 694:17-23, 1993.

I41. Baum, B.J. Dental and oral disorders. In: Abrams, W.B. and Berkow, R. (eds). Merck Manual of Geriatrics, 2nd edition. Rahway, NJ: Merck, pp. 618-630, 1995.

I42. Baum, B.J. Response to the dental scientist model. J. Dent. Educ. 57:356-358, 1993.

I43. Baum B.J., Dai, Y., Hiramatsu, Y. and Ambudkar, I.S. Receptor coupling mechanisms which regulate salivary fluid secretion. In:Murakami, M., Seo, Y. and Ishikawa, T. (eds). Salivary secretion: control and mechanisms. Okazaki, Japan: Kenbun Printing Ltd., pp.21-24, 1992.

I44. Kaplan, M.D. and Baum, B.J. The functions of saliva. Dysphagia 8:225-229, 1993.

I45. Kaplan, M.D., Wu, A.J., Baum, B.J. and Fox, P.C. Clinical assessment of salivary function. In: Martinez, J.R. (ed). US/Latin American workshop on salivary research. Albuquerque, NM: C & A Press, pp.153-159, 1993.

I46. Baum, B.J. New technologies to diagnose and treat soft tissue lesions and salivary glands. J. Amer. Dent. Assoc. 125:26S-30S,1994.

I47. Baum, B.J., Fox, P.C. and Neumann, R.D. The salivary glands. In: Harbert, J., Eckelman, W. and Neumann, R.D. (eds). Textbook of nuclear medicine, New York: Thieme Medical Publishers, pp. 439-444, 1996.

I48. Baum, B.J. Oral health in the elderly patient. Is. J. Dent. Med. 10:7-10, 1993.

I49. Drummond, J.R., Newton, J.P. and Baum, B.J. The ageing mouth. In: Yemm, R., Drummond, J.R. and Newton, J.P. (eds). Colour atlas and textbook of dental care for the elderly. London: Mosby-Wolfe, pp. 41-52, 1995.

I50. Baum, B.J. Salivary glands & chemosensory disorders: State of the art. In: Millard, H.D. and Mason, D.K. (eds). 2nd World Workshop in Oral Medicine, Univ. of Michigan Continuing Dental Education, Ann Arbor, MI, pp. 268-272, 1995.

I51. Baum, B.J. and O'Connell, B.C. The impact of gene therapy on dentistry. J. Amer. Dent. Assoc. 126:179-189, 1995.

I52. Baum, B.J. Making oral health a part of total health. Dental Abstracts 41:4-5, 1996.

I53. Baum, B.J. Oral health for the older patient. J. Amer. Geriatrics Society. 44:997-998, 1996.

I54. Baum, B.J. Perspectives from dental research. J. Dent. Educ. 60:889-890, 1996.

I55. Baum, B.J., Delporte, C., Park, C., Zheng, C., Kagami, H., Lillibridge, C.D., Braddon, V.R., Lancaster, H.E., He, X. and O'Connell, B.C. Adenoviral-mediated gene transfer: a tool for addressing biological and clinical problems of salivary glands. In: Dinudom, A. and Komwatana, P. (eds). Studies in honor of John Atherton Young. Glebe, NSW, Australia: Fast Books. pp. 39-42, 1996.

I56. O'Connell, B.C. and Baum, B.J. Gene transfer to salivary glands. In: Cohen, M.M., Jr. and Baum, B.J. (eds). Studies in stomatology and craniofacial biology. Amsterdam: IOS Press. pp. 615-631, 1997.

I57. Baum, B.J. The dental curriculum. What should be new in the 21st century. J. Public Health Dent. 56:286-290, 1996.

I58. Baum, B.J., Fox, P.C. and Tabak, L.A. Columbia, salivary research, plus Irwin Mandel equals a special combination for dentistry. J. Dent. Res. 76:631-633, 1997.

I59. Baum, B.J. The absence of a culture of science in dental education. Eur. J. Dent. Educ. 1:2-5, 1997.

I60. Delporte, C. and Baum, B.J. Preclinical and biological studies using recombinant adenoviruses encoding aquaporins 1 and 5. Eur. J. Morphol. 36:118-122, 1998.

I61. Baum, B.J. Establishing a proof of principle: an oral application of gene therapy. J. Israeli Dental Assoc. 15:7-10, 1998.

I62. Baum, B.J., Atkinson, J.C., Baccaglini, L., Berkman, M.E., Brahim, J.S., Davis, C., Lancaster, H.E., Marmary, Y., O'Connell, A.C., O'Connell, B.C., Wang, S., Xu, Y., Yamagishi, H. and Fox, P.C. The mouth is a gateway to the body: gene therapy in 21st-century dental practice. J. Calif. Dent. Assoc. 26:455-460, 1998.

I63. Fox, P.C., Brennan, M., Pillemer, S., Radfar, L., Yamano, S. and Baum, B.J. Sjogren's syndrome: a model for dental care in the 21st century. J. Amer. Dent. Assoc. 129:719-728, 1998.

I64. Baum, B.J. Re-engineering the function of salivary glands. In: Guggenheim, B., and Shapiro, S. (eds). Oral Biology at the turn of the century. Basel: S. Kaiger pp. 247-252, 1998.

I65. Baum, B.J., Wang, S., Cukierman, E., Delporte, C., Kagami, H., Marmary, Y., Fox, P.C., Mooney, D.J. and Yamada, K.M. Re-engineering the functions of a terminally-differentiated epithelial cell in vivo. Ann. NY Acad. Sci. 875: 294-300, 1999.

I66. Baum, B.J. and O'Connell, B.C. The need to introduce gene therapy in the dental curriculum. Eur. J. Dent. Educ. 3:49-51, 1999.

I67. Baum, B.J. and Wellner, R.B. Receptors in salivary glands. In: Garrett, J. R., Ekstrom, J., and Anderson, L.C. (eds). Neural mechanisms in salivary secretion. Basel, Karger, pp. 44-58, 1999.

I68. Baum, B.J. and O'Connell, B.C. In vivo gene transfer to salivary glands. Crit. Rev. Oral Biol. Med. 10:276-283, 1999.

I69. Hoque, A.T.M., Panyutin, I.G. and Baum, B.J. Use of triplex forming oligonucleotides and adenoviral constructs for studying the regulation of gene expression. Methods: a companion to Methods in Enzymology 18: 266-272, 1999.

I70. Baum, B.J., Goldsmith, C.M., Hoque, A.T.M.S., Wellner, R.B., Baccaglini, L., Ding, C., Yamano, S., Zheng, C., Aframian, D. and O'Connell, B.C. Salivary glands as a model for craniofacial applications of gene transfer. Int. J. Oral Maxillofac. Surg. 29:163-166, 2000.

I71. Baum, B.J. Crowning achievements in dentistry. The Lancet (special volume) 354: 12, 1999.

I72. Yamano, S. and Baum, B.J. Prospects for gene-based immunopharmacology in salivary glands. Japanese J. Pharmacol. 82: 281-286, 2000.

I73. Baum, B.J. and Mooney, D.J. The impact of tissue engineering on dentistry. J. Amer. Dent. Assoc. 131: 309-318, 2000.

I74. Baum, B.J. Prospects for re-engineering salivary glands. Adv. Dent. Res. 14: 84-88, 2000.

I75. Shenkin, J.D. and Baum, B.J. Oral health and the geriatrician. J. Amer. Geriatrics Soc. 49: 229-230, 2001.

I76. Hoque, A.T.M.S., Yamano, S., Baccaglini, L. and Baum, B.J. Using salivary glands as a tissue target for gene therapeutics. J. Drug Targeting 9:485-494, 2001.

I77. Simmons, R.K. and Baum, B.J. Transferring genes to salivary glands. J. Dent. Educ. 65:907-910, 2001.

I78. Atkinson, J.C. and Baum, B.J. Salivary enhancement: current status and future therapies. J. Dent. Educ. 65:1096-1101, 2001.

I79. Baum, B.J., Kok, M., Tran, S.D. and Yamano, S. The impact of gene therapy on dentistry: a re-visit after six years. J. Amer. Dent. Assoc. 133:35-44, 2002.

I80. Ship, J.A., Pillemer, S.R. and Baum, B.J. Xerostomia and the geriatric patient. J. Amer. Geriatrics Soc 50:535-543, 2002.

I81. Baum, B.J., Wellner, R.B. and Zheng, C. Gene transfer to salivary glands. Int. Rev. Cytol. 213:93-146, 2002.

I82. Vitolo, J.M. and Baum, B.J. The use of gene transfer for the protection and repair of salivary glands. Oral Diseases 8:183-191, 2002.

I83. Baum, B.J. Can biomedical science be made relevant in dental education? A North American perspective. Eur. J. Dent. Educ. 7: 49-55, 2003.

I84. Baum, B.J. Biomedical research, oral medicine, and the future. Oral. Surg. 94: 141-142, 2002.

I85. Nagler, R.M. and Baum, B.J. Prophylactic treatment reduces the severity of xerostomia following radiation therapy for head and neck cancer. Arch. Otolaryngol. Head Neck Surg. 129: 247-250, 2003.

I86. Baum, B.J. Expanding horizons: modern science enters the mouth. J. Israeli Dental Assoc. (in press).

I87. Voutetakis, A., Wang, J. and Baum, B.J. Utilizing endocrine secretory pathways in salivary glands for systemic gene therapeutics. J. Cell. Physiol. (in press).

I88. Baum, B.J., Goldsmith, C.M., Kok, M.R., Lodde, B.M., van Mello, N.M., Voutetakis, A., Wang, J., Yamano, S. and Zheng, C. Advances in vector-mediated gene transfer. Immunol. Lett. (in press).

Edited publications:

E1. Kiyak, H.A., Baum, B.J. and Levy, B.M. (eds.). A working research conference on geriatric dentistry: Current issues and future directions. In: Gerodontology Vol. 3, Numbers 1 and 2, 1984.

E2. Baum, B.J. and Fox, P.C. (eds.) Proceedings: Conference on Evaluation and Management of Salivary Gland Dysfunction. In: J. Dent. Res. (Special Issue) Vol. 66: 622-708, 1987.

E3. Baum, B.J. (ed). Special Issue: Oral, Salivary, and Dental C auses and Results of Swallowing Problems. In: Dysphagia Vol. 4, No. 2, 1990.

E4. Baum, B.J. Oral biology editorial overview. Curr. Opinion Dent. 1:793-794, 1991.

E5. Baum, B.J. (ed). Clinics in Geriatric Medicine: Oral and dental problems in the elderly. W.B. Saunders Co., Philadelphia, 1992.

E6. Baum, B.J. (ed). Proceedings: 13th International Conference on Oral Biology Tissue Repair. In: Adv. Dent. Res. Vol. 9, No. 3, 1995.

E7. Cohen, M.M. Jr and Baum, B.J. (eds) Studies in Stomatology and Craniofacial Biology. IOS Press, Amsterdam, The Netherlands, 1997.

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ARTICLES

SALIVARY GLAND TRANSPLANTATION: A CANINE MODEL

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Impaired salivary function with resultant severe dryness of the mouth, or xerostomia, may occur in association with a variety of systemic disorders or therapies. No adequate treatment exists for this debilitating condition, which impedes normal oral function, in particular alimentation and phonation. This study explores the feasibility of salivary gland autotransplantation, using a canine model. A salivary gland with its duct and surrounding blood vessels still attached was excised and reimplanted in the dog's thigh by anastomosing the graft's blood vessels to the femoral artery and vein. The duct was sutured to an artificial orifice cut in the thigh's skin, from which the saliva was collected. Salivary secretion was induced by a single intravenous bolus of pilocarpine (5 mg). Preoperative (normal) salivation was measured by collecting saliva from the gland in situ. Periodic functional studies showed normal saliva production during the first month after grafting, after which the salivary flow was reduced by 35% over the next 2 months. This reduction was interpreted as a sign of disuse atrophy resulting from the lack of autonomic innervation. To overcome this impediment, oral pilocarpine (5 mg/day) was administered to the recipient dog, after which normal levels of saliva were excreted through the graft during the 3-month follow-up period. The quality of the graft saliva was assessed by its protein and electrolyte levels, which showed close to normal values.

Diminished salivary flow to the point of extreme dry mouth, xerostomia, is usually a sequela of irradiation to the head and neck region or certain drug therapies, or it may be a symptom in systemic diseases such as Sjögren's syndrome. In the Western world, including the United States, there is an increasing prevalence of salivary hypofunction (*1, 2*). Although not a fatal condition, the quality of life of xerostomic patients is gravely reduced because two basic human functions, alimentation and oral communication, are severely impaired. Lack of saliva prevents oral tissue lubrication, thereby obstructing solid food bolus formation and impeding phonation. Scarce or no salivary output will lower antimicrobial protection and attack oral mucosal membrane integrity (*3*).

Two main approaches have been used in the attempt to alleviate the symptoms of xerostomia: systemic therapy and the use of artificial saliva (saliva substitutes). The systemic treatment, which is empirical in nature, is directed at the underlying cause of the phenomenon and includes antimalarial drugs, steroids, cytotoxic agents, interferon α-2, and, especially in Sjögren's syndrome patients, antirheumatic agents (*4–9*). In clinical, controlled studies using these agents, the outcome with respect to salivary function was equivocal; furthermore, numerous side effects were reported, making such an approach worthless in the majority of cases, especially in view of the minimal relief experienced by the patients. Another systemic mode of treatment of the dry mouth syndrome is aimed directly at the salivary dysfunction and consists of systemic therapy with sialagogues to promote the secretory potential of the residual gland. A number of such salivary stimulants have been studied in clinical trials (*10–13*), but none of them succeeded in increasing considerable salivary output, and some were even associated with gastrointestinal disorders. The few agents that were able to elicit salivary secretion, albeit on a modest scale, were pilocarpine and some traditional medicaments (*14–19*).

It should be stressed that none of the patients in the studies cited above had total glandular atrophy, because all displayed functional salivary remnants. Administration of any systemic drugs to patients who underwent head and neck irradiation or those who suffer from severe Sjögren's syndrome with a total lack of functional gland parenchyma is to no avail, because the rationale for their use is based on augmentation of remaining glandular tissue.

The use of artificial saliva to overcome the problem of salivary dysfunction is aimed at patients without functional glandular tissue. The basic components of most artificial saliva compounds are methylcellulose, sorbitol, ions including fluoride, and preservatives; some preparations contain porcine-derived mucin. However, the currently available products are far from satisfactory in their efficacy and may even have unwarranted side effects, such as irritation of the mucosal tissue and unpleasant taste.

In light of the success of transplantation as a therapy for a

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variety of end-stage organ failure, transplantation of the salivary gland could be an effective treatment modality for certain patients suffering from severe xerostomia. Within this context, it is worthwhile mentioning the use of pancreas transplantation in diabetic patients and the high rate of functioning glands for a period of several years after transplantation. The exocrine function of allotransplanted pancreas in humans has been reported to be 75% and 60% at 1- and 5-year follow-up, respectively (*20–22*). The fact that pancreatic exocrine activity can be preserved indicates the feasibility of exocrine gland transplantation, making salivary gland transplantation a workable concept. The present study aimed at establishing an animal model for salivary gland transplantation to assess the feasibility of saliva production by a transplanted gland.

MATERIALS AND METHODS

All animal procedures employed in this study were approved by the Institutional Committee for Animal Care and Experimentation. A total of 10 mongrel male dogs weighing 23 to 30 kg was used, 4 in preliminary studies and 6 in the transplantation model itself. Of the latter, five procedures were autotransplant, and in one instance an allograft was performed. The preliminary experiments were conducted to explore the basic anatomy relevant to the transplantation procedures. The anatomy of the gland and its environs were unraveled by means of dissection and exploration of the major salivary glands and their relationship to other structures and the mode of their blood supply. Surgery was carried out in anesthetized and intubated animals. Anesthesia was induced by intravenous injection of pentothal (60 mg/kg) followed by intratracheal intubation. Ventilation was maintained with 2 to 5% halothane in O_2/NO_2. Functional studies, i.e., saliva collection, were always performed on anesthetized and intubated dogs.

The transplantation model will be described in detail in *Results*. In brief, the mandibular salivary gland was explanted and autografted or allografted into the inguinal fossa, using the femoral vessels for vascular anastomoses. The salivary duct was exteriorized at the skin level, creating a salivary stoma in the thigh. To minimize cold ischemia time, the femoral vessels were prepared for anastomosis before gland explantation.

Perioperative treatment included daily intramuscular injections of penicillin (1×10^6 units) and streptomycin (1 g) for 10 days. The dogs were allowed to drink and eat ad libitum; during the first 4 postoperative days, food was supplemented with liquid nutrition (Ensure, Ross Laboratories, Columbus, OH). Two of the five autotransplanted dogs received 5 mg/day pilocarpine in the form of an oral tablet (prepared by us) during the entire follow-up period (Sigma, St. Louis, MO).

To obtain reference (control) values, the function of the mandibular salivary gland in situ was analyzed 2 to 3 days before explantation. The orifice of the gland located at the lingual frenulum in the floor of the mouth was cannulated using a 17-G polyethylene tube. To stimulate salivation, pilocarpine (5 mg in 1 ml of saline) was injected intravenously; after this, the saliva, which started to flow within less than 1 min, was collected for 10 min into 1-min tubes. Total volume and flow rate were recorded. Samples were weighed and stored at −4°C. In a similar manner, the function of the transplanted glands was studied for 3 months after transplantation on a weekly basis.

Saliva quality was assessed by assaying total protein, sodium, and potassium concentrations in each sample. Total protein levels were determined by ultraviolet absorption at 215 nm using a Uvikon 810 spectrophotometer (Kontron, Zurich, Switzerland); electrolyte concentration were measured by means of atomic absorption (Perkin-Elmer 403, Norwalk, CT).

RESULTS

The experimental model of salivary gland transplantation. The preliminary studies determined the choice of the gland to be explanted. The submandibular gland was elected as the transplant organ because of its superficial location, which permits easy access, and because of its solid encapsulated texture. It was exposed after a Y-shaped incision was made around the mandibular angle (Fig. 1). One area of the incision runs superiorly along the posterior border of the mandible, another along its lower border, and the third part runs posteriorly along the dog's neck. After developing two skin flaps, the venous drainage was ascertained (Fig. 2). Two to three tiny glandular veins draining into the linguofacial, maxillary, or jugular veins were found. Venous drainage of the gland transplant was based on the latter large veins, which were fully dissected and mobilized. The gland was subsequently separated from the underlying stylohyoid and sternomastoid muscles in a cranial to caudal direction. The anterior aspect of the gland was separated from the ascending ramus of the mandible, exposing the arterial supply of the gland at its superior-anterior aspects. Two glandular arteries, both branches of the maxillary artery, were found (Fig. 2). The main glandular duct originating at the anterior and medial aspect of the gland was identified and dissected. It was fully freed until it entered the floor of the mouth.

At this stage, the glandular graft was ready for explantation (Fig. 3). The linguofacial and maxillary veins were ligated and transected close to the mandibular ramus. The jugular vein was transected 1.5 cm distal to its bifurcation. The maxillary artery was transected proximal and distal to the takeoff glandular branches, and the duct was transected at the distal end. The graft was removed and immediately rinsed with a cold heparinized saline solution (0.05%), as follows: a cannula was inserted into the maxillary artery and the solution was flushed through the gland using 1 m gravity force until it was pale in color. Systemic heparin was administered to neither the donor nor the recipient dogs. During the entire period of devascularization and until grafting, the

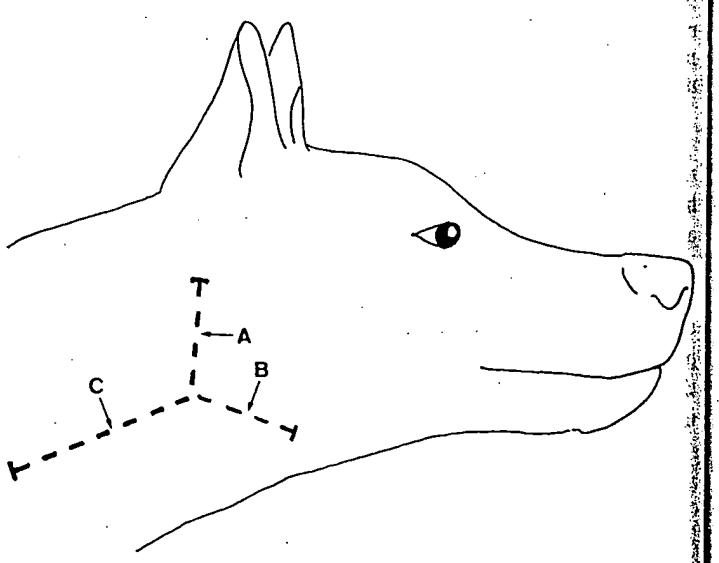

FIGURE 1. Schematic drawing of the dog's neck illustrating the incision intended for harvesting the mandibular salivary gland graft. (A) Superior incision along the posterior border of the mandible; (B) incision along the lower mandibular border; (C) posterior incision.

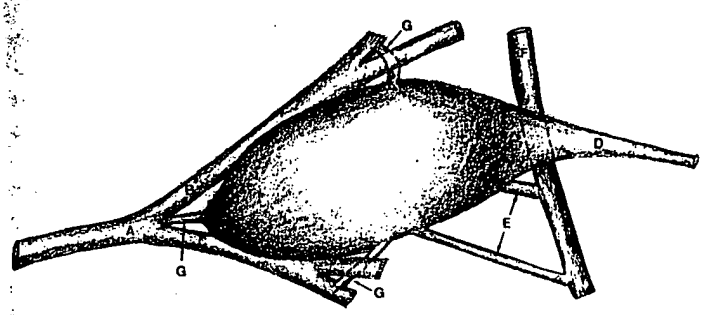

FIGURE 2. Schematic drawing of a right mandibular canine salivary gland (donor gland). (A) External jugular vein; (B) maxillary vein; (C) linguofacial vein; (D) mandibular duct; (E) glandular arteries; (F) maxillary artery; (G) glandular veins.

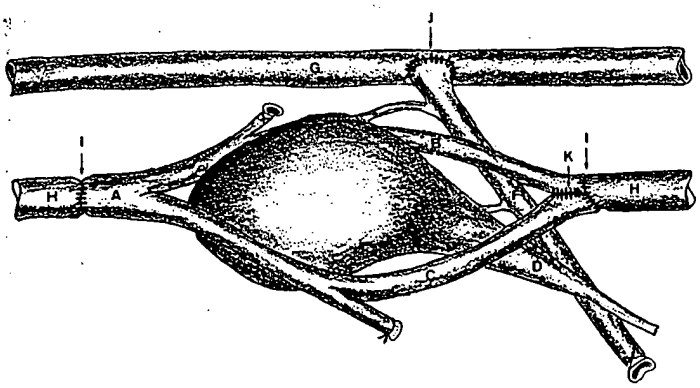

FIGURE 3. Schematic drawing of the prepared graft anastomosed to the femoral blood vessels. (A) External jugular vein; (B) maxillary vein; (C) linguofacial vein; (D) mandibular duct; (F) maxillary artery; (G) femoral artery.

gland was kept on ice. The distal ends of the linguofacial and maxillary veins were anastomosed (side-to-side) to form one main trunk that served as the distal end of the graft's vein, and the jugular vain formed its other end. (Fig. 3).

For the grafting procedure, blood flow from the femoral vein was blocked by two vessel loops. A portion of the vein, comparable in length to that of the donor vein, was excised. The proximal and distal ends of the graft's veins were anastomosed (end-to-end) to the prepared (femoral) recipient vein. The glandular artery was anastomosed (end-to-side) to the femoral artery after temporary ligation of the femoral artery (Fig. 3). The skin near the graft was perforated, and the distal end of the salivary duct was connected to the edge of the stoma to allow free salivary flow. All suturing was performed with 7–0 nylon thread.

Autotransplant functional studies. Six dogs were grafted according to the method described above. Two of the autografted glands secreted saliva immediately upon surgery; this ceased, however, quite soon thereafter. Surgical exploration of these two transplants showed graft necrosis resulting from vascular thrombosis. Three other autografts secreted substantial amounts of saliva during the follow-up period. The one allotransplanted gland was studied on a daily basis; it showed normal salivation during the first 4 postoperative days, followed by a gradual decrease until on the 8th day no saliva was obtainable at all from the graft.

Figure 4 presents the total amount of saliva collected (after pilocarpine stimulation) from one autograft during the

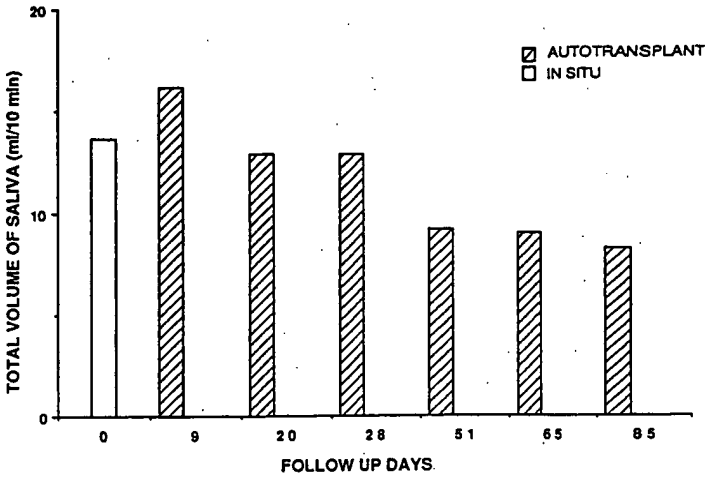

FIGURE 4. Autotransplant salivation in the dog receiving no pilocarpine treatment.

3-month follow-up period. In the first postoperative month, 13 to 16 ml/10 min saliva was excreted, approaching the control volumes of the same gland. Considerable volumes, albeit 33 to 39% below normal, were still measured on days 51, 65, and 85.

Salivation flow rate, another parameter of salivary gland function, was studied after intravenous pilocarpine stimulation. The autotransplanted gland deviated from the normal with regard to this parameter. In situ, the flow rate decreased gradually, i.e., from 3 ml in the first minute, to 2.5 ml in the second minute, to about 1 ml after 6 to 8 min. After transplantation, the flow rate showed a plateau, remaining at 1 to 1.5 ml throughout the collection time (Fig. 5).

This phenomenon of an equal flow rate was presumed to be the result of changes occurring in a gland lacking continuous stimulation because of deficient innervation. To overcome the problem of an inadequate supply of nerve fibers, a daily muscarinic stimulus (the cholinomimetic alkaloid pilocarpine) was administered to two additional autotransplanted dogs during the follow-up period. It resulted in continuously high levels of saliva (Fig. 6), with a normal pattern of flow rate at each measuring point. Figure 7 is a represen-

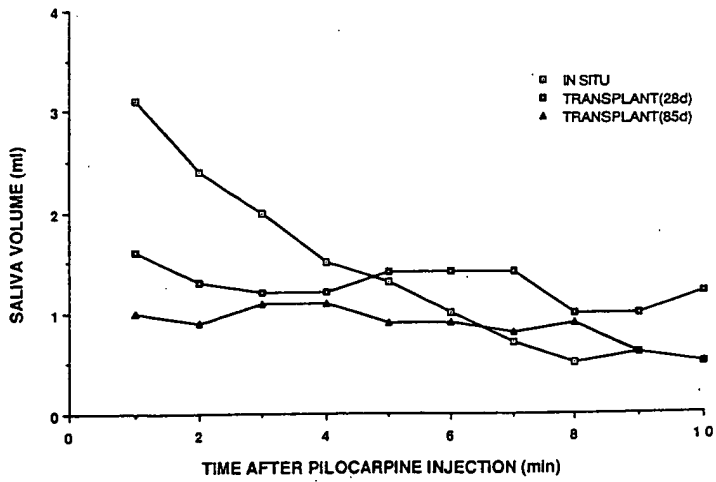

FIGURE 5. Autotransplant flow rate measurements after 28 and 85 days after transplantation in the dog receiving no pilocarpine treatment. In situ (control) salivary flow rate is also shown.

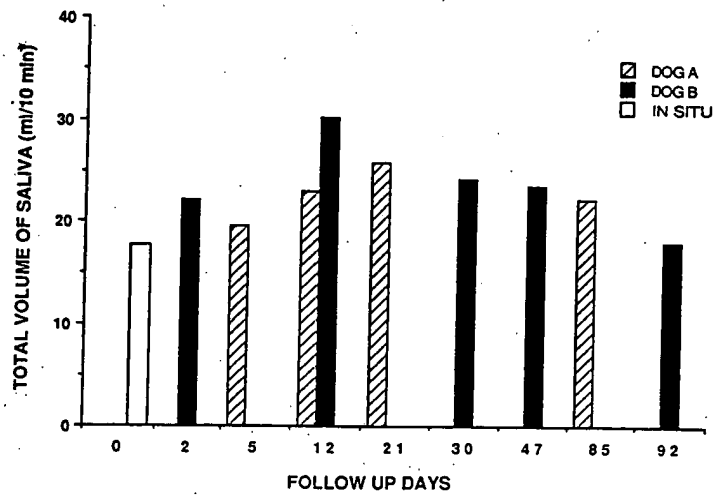

FIGURE 6. Autotransplant salivation during the 92 experimental days in the two recipient dogs treated continuously with oral pilocarpine.

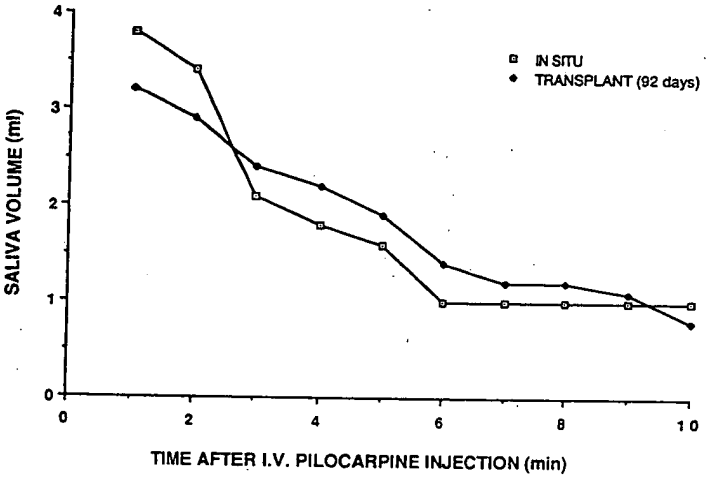

FIGURE 7. Autotransplant flow rate measured on day 92 after surgery in one of the dogs receiving oral daily pilocarpine therapy during the entire follow-up period.

tative example of the salivary flow rate of an autotransplanted gland on day 92 compared with its rate measured in situ.

Salivary protein, potassium, and sodium concentrations, representative parameters of saliva quality, were similar in both in situ and transplanted gland. Thus, for example, in the non-pilocarpine-treated dog monitored longitudinally, the salivary protein level amounted to 4.2 mg/ml on day 85 compared with the control value of 4.4 mg/ml.

DISCUSSION

To the best of our knowledge, transplantation of the salivary gland has never been presented in the medical literature. However, based on the encouraging results with pancreas transplantation in diabetic patients (*20–22*), we proffer the concept of salivary gland transplantation as a therapeutic modality in patients with severe xerostomia.

We envisage salivary gland transplantation as a treatment modality in patients afflicted with severe xerostomia resulting from total lack of saliva caused by Sjögren's syndrome or after head and neck irradiation. To this end, it seems logical to implement allograft transplantation by using human submandibular glands implanted into the submandibular triangle located in close proximity with the floor of the recipient mouth. The human submandibular gland lends itself extremely well to transplantation because of its encapsulated nature, sizable blood vessels, and long excretory duct. Furthermore, the anatomy of the gland and that of the recipient region is quite familiar to surgeons of various disciplines. Immunosuppressive drugs are indicated in these instances because of the high therapeutic index (prescription of these drugs for treating the hyposalivation in Sjögren's syndrome patients was discontinued because of their limited and transient efficacy). Patients with head and neck cancer treated by irradiation will be eligible for salivary gland allotransplantation and subsequent immunosuppressive treatment when they are considered cured of their malignancy, in keeping with the rationale applied in other organ transplantations for cancer patients.

The first step toward achieving this goal in humans had to be the establishment of an animal model to study the functional performance of a transplanted salivary gland. Dogs were considered the most suitable to serve as the experimental animals because of their sizable salivary glands and related blood vessels. The inguinal fossa was chosen as the recipient site because of the ample blood vessels and the easy access for the grafting and transplant saliva collection procedures. The autograft modality was used at this stage of model development to avoid the problem of rejection. To overcome the technical difficulties of anastomosing the donor's very small veins and arteries to the recipient site, a strategy of harvesting the gland together with the more distally placed vessels (segments of the linguofacial, maxillary, and jugular veins and the maxillary artery) was used.

The lack of innervation in the autotransplanted glands may have been the underlying reason for the 30% reduced values of salivary flow obtained in the first autograft experiment. It is assumed that the deficient autonomic stimulation resulted in a down-regulation of acinar cell receptors in the autotransplanted gland (Figs. 4 and 5). To counteract this phenomenon, oral pilocarpine was administered daily to the animals in the subsequent autotransplant experiments (Figs. 5 and 6) to provide continuous muscarinic receptor stimulation (*14*).

By using these methods, substantial amounts of saliva approximating normal volumes were produced by the transplanted glands throughout the 3 months of the postsurgical period. No importance should be attached to the experimental period of 3 months, because this was a purely arbitrary decision. It is of interest that one grafted dog that had been allowed to stay in the animal house with no special care, i.e., daily pilocarpine administration, excreted normal amounts of saliva after 14 months, indicating possible restoration of autonomic innervation. The slight reduction in salivary gland autotransplant function is similar to that noted for the exocrine secretion in human pancreatic allografts (*20*).

It should be stressed that the experimental salivation described in this study is a stimulated procedure by means of intravenous pilocarpine, a well accepted method to study salivary gland function in both humans and animals (*23, 24*). The parameter characterizes glandular potential and reflects the gland's status. Under normal in vivo conditions, resting saliva is low and higher volumes are secreted only upon

gustatory or other stimulation (*23, 24*). Resting saliva cannot be measured in anesthetized animals. In possible future human salivary gland transplantation to ameliorate insufficient salivation, administration of pilocarpine on a daily basis might be the appropriate stimulatory method to overcome the deficient autonomic innervation. Such treatment has been shown to have beneficial effects in moderately xerostomic patients (*17*).

Normal saliva is a watery solution rich in electrolytes and biologically highly potent proteins, such as mucins, cystatin-rich phosphoproteins, enzymes, immunoglobulins, proline-rich proteins, and several other important constituents. In the present study, the concentrations of only total proteins and two representative electrolytes were assayed. We termed this transplant product "saliva," although admittedly more detailed analyses are necessary for a precise definition. Nevertheless, even if not all salivary components prove to be present in the graft saliva, one cannot disregard the advantage of improved secretion to whatever degree in alleviating the immense discomfort of severe xerostomia.

In conclusion, we believe that the present investigation succeeded in establishing a useful experimental model of salivary gland transplantation. Further animal studies are needed to explore, among other topics, allograft rejection and explant preservation, with the ultimate aim of salivary gland transplantation in humans.

REFERENCES

1. Loe H. Welcome. J Dent Res 1987; 66: 621.
2. Levine MJ, Aguirre A, Hatton MN, Tabak LA. Artificial salivas: present and future. J Dent Res 1987; 66: 693.
3. Mandel ID. The function of saliva. J Dent Res 1987; 66: 52
4. Fox RI. Treatment of the patient with Sjögren's syndrome. Rheum Dis Clin North Am 1992; 18: 699.
5. Tabbara KF, Frayha RA. Alternate-day steroid therapy for patients with primary Sjögren's syndrome. Ann Ophthalmol 1983; 15: 358.
6. Skopouli FN, Jagiello P, Tsifetaki N, Moutsopoulos HM. Methotrexate in primary Sjögren's syndrome. Clin Exp Rheumatol 1996; 14: 555.
7. Fox RI. Antimalarial drugs: mechanisms of action in autoimmune disease and prospects for drug development. Clin Immunother 1995; 4: 219.
8. Kruize AA, Hene RJ, Kallenberg CGM, et al. Hydroxychloroquine treatment for Sjögren's syndrome: a two-year double-blind crossover trial. Ann Rheum Dis 1993; 52: 360.
9. Ferraccioli GF, Salaffi F, De Vita S, et al. Interferon alpha-2 (IFalpha2) increases lacrimal and salivary function in Sjögren's syndrome patients. Preliminary results of an open pilot trial versus OH-chloroqui. Clin Exp Rheumatol 1996; 14: 367.
10. Fox PC. Systemic therapy of salivary gland hypofunction. J Dent Res 1987; 66 (special issue): 689.
11. Prause JV, Frost-Larsen K, Hoj L, Isager H, Manthorpe R. Lacrimal and salivary secretion in Sjögren's syndrome: the effect of systemic treatment with bromhexine. Acta Ophthalmol 1984; 62: 489.
12. Epstein JB, Decoteau WE, Wilkinson A. Effect of sialor in treatment of xerostomia in Sjögren's syndrome. Oral Surg 1983; 56: 495.
13. Schiodt M, Oxholm P, Jacobsen A. Treatment of xerostomia in patients with primary Sjögren's syndrome with sulfarlem. Scand J Rheumatol 1986; 61: 250.
14. Greenspan D, Daniels TE. The use of pilocarpine in post-radiation xerostomia [Abstract 1319]. J Dent Res 1979; 58: 420.
15. Fox PC, Van Der Ven PF, Baum BJ, Mandel ID. Pilocarpine for the treatment of xerostomia associated with salivary gland dysfunction. Oral Sug 1986; 61: 243.
16. Fox PC, Van Der Ven PF, Baum BJ. Treatment of salivary gland hypofunction with sustained-release pilocarpine HCl [Abstract 563]. J Dent Res 1986; 65: 232.
17. Johnson JT, Ferrenti GA, Nethery WJ, et al. Oral pilocarpine for postirradiation xerostomia in patients with head and neck cancer. N Engl J Med 1993; 329: 390.
18. Ohno S, Suzuki T, Dohi Y. Effect of bakumondo-to on salivary secretion in patients with Sjögren's syndrome. Jpn J Rheumatol 1992; 4: 91.
19. Goto M. Long-term effects of Japanese herb medicine, bakumondo-to (mai-men-dong-tang), for the treatment of sicca syndrome. Int J Immunother 1994; 10: 173.
20. Sutherland DER, Kendal D, Gaetz FC, Najarian JS. Pancreas transplantation in humans. In: Wayne Flye M, ed. Principles of organ transplantation. London: Saunders, 1989: 364.
21. Prieto M, Sutherland DER, Fernandez-Cruz L, Heil J, Najarian JS. Experimental and clinical experience with urine amylase monitoring for early diagnosis of rejection in pancreas transplantation. Transplantation 1987; 43: 73.
22. Bromberg JS, Punch JD, Merion RM, et al. Transplantation and immunology. In: Greenfield LJ, Mulholland M, Oldham KT, Zelenock JB, Lillemoe KD, eds. Surgery: scientific principle and practice. 2nd ed. Philadelphia-New York: Lippinocott-Raven, 1997: 527.
23. Mandel ID, Katz R, Zengo A, et al. The effect of pharmacologic agents on salivary secretion and composition in man: I. Pilocarpine, atropine and anticholinesterases. J Oral Ther Pharmacol 1968; 4: 192.
24. Marmary Y, Okon E, Katz J, Shiloni E. Effect of interleukin-2 on salivary gland morphology and function in mice. Lymphokine Cytokine Res 1994; 13: 197.

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Original Contribution

DESIGN AND FABRICATION OF BIODEGRADABLE POLYMER DEVICES TO ENGINEER TUBULAR TISSUES

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☐ **Abstract – Engineering new tissues by transplanting cells on polymeric delivery devices is one approach to alleviate the vast shortage of donor tissue. However, it will be necessary to fabricate cell delivery devices that deliver cells to a given location and promote the formation of specific tissue structures from the transplanted cells and the host tissue. This report describes the design and fabrication of a polymeric device for guiding the development of tubular vascularized tissues, which may be useful for engineering a variety of tissues including intestine, blood vessels, tracheas, and ureters. Porous films of poly (D,L-lactic-*co*-glycolic acid) have been formed and fabricated into tubes capable of resisting compressional forces in vitro and in vivo. These devices promote the ingrowth of fibrovascular tissue following implantation into recipient animals, resulting in a vascularized, tubular tissue. To investigate the utility of these devices as cell delivery devices, enterocytes (intestinal epithelial cells) were seeded onto the devices in vitro. Enterocytes were found to attach to these devices and form an organized epithelial cell layer. These results suggest that these devices may be an appropriate delivery vehicle for transplanting cells and engineering new tubular tissues.**

☐ **Keywords – Tissue engineering; Biodegradable polymers; Tubular tissues.**

INTRODUCTION

A significant factor limiting the therapies of organ transplantation and tissue reconstruction is the lack of suitable donor tissue (1). Our laboratory has focused on the creation of new tissue substitutes with selective cell transplantation on biodegradable synthetic polymers (7,14) to alleviate this problem. This approach involves isolating cells from a portion of donor tissue and attaching them to polymer scaffolds. The cell-polymer devices are subsequently implanted into appropriate sites to promote cell growth and reorganization into the desired tissue structure. Utilizing this approach we have engineered liver, cartilage, bone, and urothelial tissue in animal models (2,12,13,16). The polymer template serves both as a delivery vehicle for transplanted cells and as a template to guide the development of tissue structure. Furthermore, the use of biodegradable polymers allows one to have a scaffold that will erode after tissue development is completed to leave an entirely natural tissue.

A major challenge in any attempt to engineer new tissue is the development of a cell delivery device that meets the requirements for the specific tissue. There are a variety of tissues with tubular structure, including intestine, blood vessels, tracheas, and ureters, and a tubular delivery device will likely be required to engineer these tissues. Engineering neointestine is of interest, for example, as loss of a large portion of small bowel leads to a state of malnutrition termed "short bowel syndrome," which, if left untreated, results in death. Parenteral nutrition, feeding with a fluid introduced through major blood vessels, improves patient survival, but long-term treatment often results in liver disease or infection, and loss of vascular access (3). The most promising alternative therapy, small bowel transplantation, is not widely performed, and expansion of this treatment to a larger percentage of patients will likely be limited by the significant problem of donor shortage (1). It may be possible to engineer neointestine and other tubular tissues if a delivery device were fabricated that was tubular, biocompatible, and allowed neovascularization of the developing tissue while preventing the invading tissue from collapsing the device.

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In this study we describe the fabrication of cell delivery devices that meet these requirements. Tubular devices were fabricated from porous films of poly (D,L-lactic-*co*-glycolic acid). The devices are biocompatible, have sufficient mechanical stability to prevent tissue collapse, and promote vascularization of the developing tissue. Furthermore, intestinal epithelial cells (enterocytes), attach to the devices and form an organized cell layer on their luminal surface. Thus, these devices may allow transplantation of specific all types, and promote the formation of a desired tissue structure.

MATERIALS AND METHODS

Materials

The 50/50 copolymer of lactic and glycolic acid (poly (D,L-lactic-*co*-glycolic acid) was purchased from Medisorb (Cincinnati, OH), chloroform from Mallinckrodt (Paris, KY), polystyrene standards from Polysciences (Warrington, PA), aluminum backed tape from Cole-Parmer (Chicago, IL), phosphate buffered saline and DMEM medium from Gibco (Grand Island, NY), Tmax film from Kodak, Lewis rats, 250–300 g, from Charles River (Wilmington, MA), calf serum from Hyclone Lab. Inc. (Logan, UT), penicillin and streptomycin from Irvine Scientific (Santa Ana, CA), and methoxyflurane from Pitman-Moore Inc. (Mundelein, IL).

Device Characterization

Devices were synthesized from a 50/50 copolymer of lactic and glycolic acid (poly (D,L-lactic-*co*-glycolic acid) with a molecular weight (M_w) of 43,400 (M_w/M_n = 1.43). Molecular weights were determined by gel permeation chromatography (Perkin-Elmer, Series 10, Newton Centre, MA), using polystyrene standards to generate a calibration curve.

For scanning electron microscopic examination, samples were gold coated using a Sputter Coater (Desk II, Denton Vacuum, Cherry Hill, NJ). An environmental scanning electron microscope (ElectroScan; Wilmington, MA) was operated at 30 kV with a water vapor environment of 5 torr to image samples. Photomicrographs were taken with Polaroid 55 film.

The porosity and pore size distribution of devices was analyzed by mercury porosimetry (Poresizer 9320, Micromeritics, Norcross, GA) using a solid penetrometer with a 5 mL bulb volume (920-61707-00, Micromeritics). The void volume and pore size distribution of polymer devices were determined as previously described (5).

To determine the yield stress and strain of the polymer films used to form tubes, they were subjected to a tensile test using an Instron model 4201 (Canton, MA). Testing was done at a constant strain rate of 0.1 mm/s, and testing was continued until the samples failed.

Thermal mechanical analysis of devices was performed with a TMA 7 (Perkin Elmer Corp.; Norwalk, CT) at a constant temperature of 37°C. A load of 100 mN was applied for 15 min, and then removed. The height of the sample was followed during compression and following release of the load. Differential scanning calorimetry was performed with a DSC 7 (Perkin Elmer Corp.; Norwalk, CT) with a heat ramp of 10°C/min.

The degradation kinetics of the formed tubes were analyzed by placing each device in 5 mL of phosphate buffered saline, pH 7.4, and incubating under static conditions at 37°C. The mass loss was analyzed by weighing lyophilized devices before and after the incubation period. The molecular weight changes of the polymer devices were analyzed using gel permeation chromatography as described above.

Implantation of Devices

Polymer constructs were implanted into the mesentery of syngeneic Lewis rats as previously described (8). The mesenteric tissue was rolled around the devices to promote tissue invasion and neovascularization of the implants from all sides. Implants were secured in place with sutures of 7-0 Maxon (Davis and Geck). Recipients of polymer devices were sacrificed on post implantation days 0, 3, 7, and 56. The implants were removed, fixed in 10% buffered formalin, and thin sections were cut from paraffin-embedded tissue. Histological sections were stained with hematoxylin and eosin. Photomicrographs were taken with Kodak Tmax film. Inhalation anesthesia with methoxyflurane was utilized in all experiments involving animals.

Cell Isolation and Attachment to Devices

Segments of small intestine (20 cm) were isolated from syngeneic Lewis rats by making a midline incision to expose the small intestine, lavaging the intestine to remove fecal debris, and excising along the mesenteric attachment. Enterocytes were nonenzymatically isolated from intestinal segments by serial incubation of the intestinal lumen with an isotonic solution containing EDTA as previously described (8). This method isolates separate fractions of villus and crypt cells. The undifferentiated, but highly proliferative crypt cells were collected, counted on a Coulter counter, and assessed for viability by trypan blue exclusion. Approximately 3×10^6 crypt cells were isolated per cm of original intestine, and the average cell viability was 77%.

To introduce cells into the polymeric delivery devices, 0.15 mL of a cell suspension containing from

$0.3\text{–}20 \times 10^6$ cells/mL was injected into the interior of each device using a 1 mL syringe and a 22 gauge needle. Devices were incubated for 24 h at 37°C in an atmosphere of 5% CO_2 to allow cell adhesion, and the caps at the ends were then cut off. To quantitate the number of adherent cells the devices were washed twice with phosphate buffered saline, and incubated with 1 mL of 0.25% trypsin for 1 h. The number of detached cells was then quantitated using a Coulter counter. Cell-polymer devices were kept in DMEM medium, containing 5% calf serum, 100 U/mL penicillin and 100 mg/mL streptomycin during the period of cell attachment.

RESULTS

Fabrication and Characterization of Polymer Devices

To fabricate cell delivery devices, a material that was biocompatible and biodegradable was desired. A rapid degradation was desired so that the chronic foreign-body inflammatory response could be minimized, along with the resulting scar tissue formation. A 50/50 copolymer of D,L lactic acid and glycolic acid was chosen to fabricate cell delivery devices, as polymers of the lactic-glycolic acid family are biocompatible (15), degrade by simple hydrolysis (10), and are FDA approved for certain applications in humans. Copolymers of D,L lactic acid and glycolic acid are amorphous (6), increasing the rate of degradation, and are relatively malleable. Differential scanning calorimetric analysis of the chosen polymer confirmed the noncrystalline nature of the polymer, as a glass transition was noted at 48°C, and no crystallization peak was observed.

Delivery devices were fabricated by first preparing porous films of the polymer, and then forming these films into tubes. Porous films were formed by casting a dilute solution of the 50/50 copolymer dissolved in chloroform into Teflon covered petri dishes. Sodium

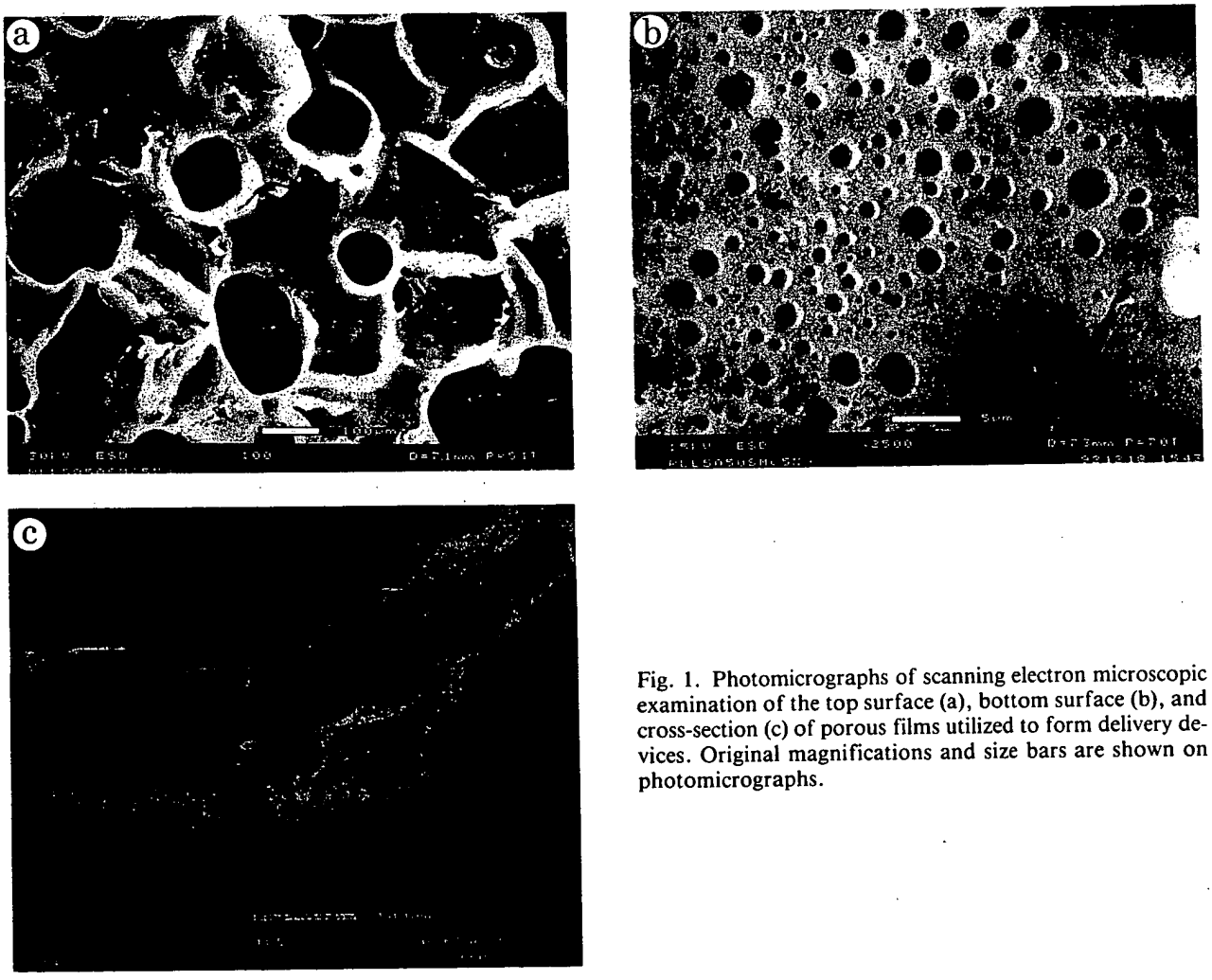

Fig. 1. Photomicrographs of scanning electron microscopic examination of the top surface (a), bottom surface (b), and cross-section (c) of porous films utilized to form delivery devices. Original magnifications and size bars are shown on photomicrographs.

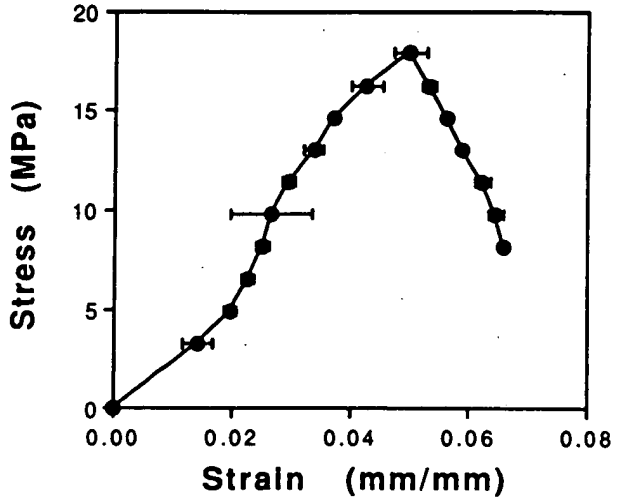

Fig. 2. A representative stress-strain diagram of porous films subjected to a constant strain rate of 0.05 mm/min at room temperature. Values are the mean ± standard deviation calculated from measurements taken on three samples of a film.

chloride particles were distributed over the solution, the chloroform was evaporated, and the salt particles leached out. This led to the formation of thin polymer films (0.31 ± 0.02 mm; $n = 6$), and scanning electron microscopic examination confirmed the highly porous nature of these films. The surface exposed to air was very rough, with large pores (100–200 μm) (Fig. 1a). In contrast, the surface exposed to the Teflon coating was much smoother, with pores ranging from 5–50 μm (Fig. 1b). Examination of a cross-section of the films indicated that the films were largely hollow, with pores corresponding in size to the salt crystals originally cast with the polymer solution (Fig. 1c). In many ways this anisotropic structure was ideal. The rougher surface could be used to form the outer surface of the tubes, as large pores are necessary to promote tissue ingrowth and vascularization of implants (17). The smoother surface could be used to form the interior of the devices, as a smooth surface would allow adherent cells to more readily form an organized monolayer than would a highly irregular surface.

To confirm the scanning electron microscopy observations, the porosity and pore size distribution of the films were quantitated using mercury porosimetry. The porosity of the devices was 89 ± 2%, and the average pore size in the films was 139 ± 62 μm (mean ± standard deviation; $n = 6$), corresponding to the size of the NaCl crystals loaded.

A strong, but malleable polymer was required to permit formation of tubes from the cast films. To confirm that the processing technique did not significantly change the malleability of the 50/50 copolymer, the maximal elongation before failure and yield stress of formed films was measured. A stress-strain diagram of the films is shown in Fig. 2, and this testing indicated that the polymers remained malleable after the solvent casting and leaching processes. The average yield strain of films was 5.2 ± 0.9% ($n = 5$), and the average yield stress was 14.1 ± 5.4 MPa. These values are in the same

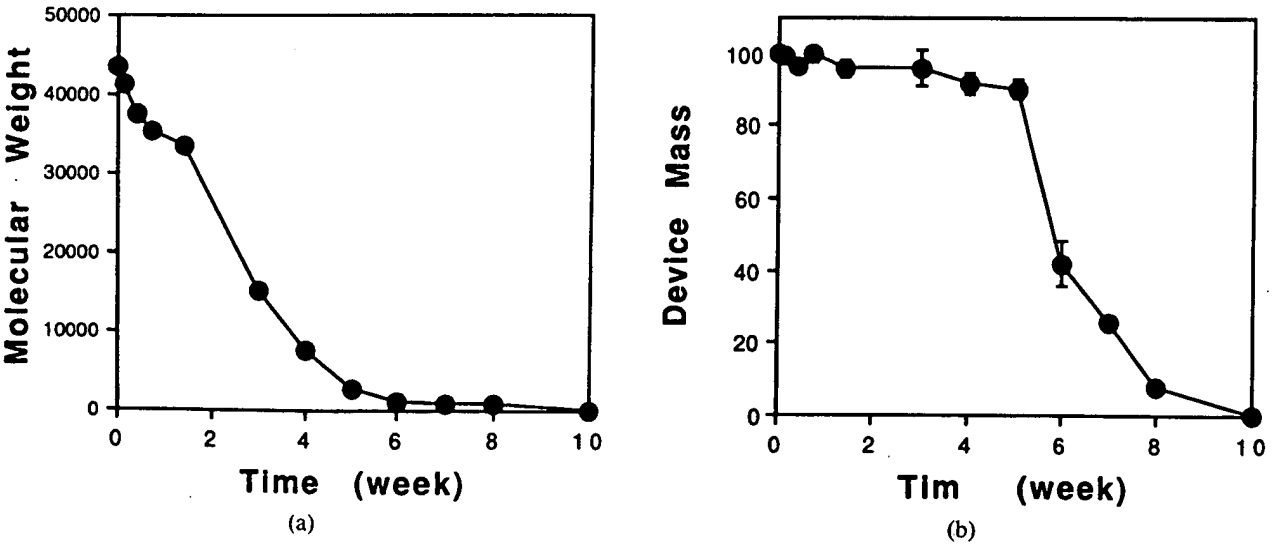

Fig. 3. Change in molecular weight (a), and mass (b) of delivery devices placed in phosphate buffered saline under static conditions at 37°C. The masses of devices were normalized to the initial mass, and values represent the mean and standard deviation of 4 devices at each time point.

range as the stock polymer utilized to form the films (9). Furthermore, the glass transition temperature of the films was the same as the stock polymer (not shown), again indicating that the processing did not significantly change the polymer.

To form tubular devices, the porous films were subsequently wrapped around Teflon cylinders, and the overlapping ends were sealed together. A design criteria for these devices is that they withstand compressional forces in vivo, thus preventing tissue collapse. Compression of devices by a force of up to 200 mN using a thermomechanical analysis system led to slight deformations (i.e., a force of 100 mN resulted in a strain of 7%), but the tubes did not collapse under this load. This deformation was mainly elastic, as the tubes returned to near the original diameter upon removal of the load. Another design criteria was that these devices degrade over a period of weeks to months follow-

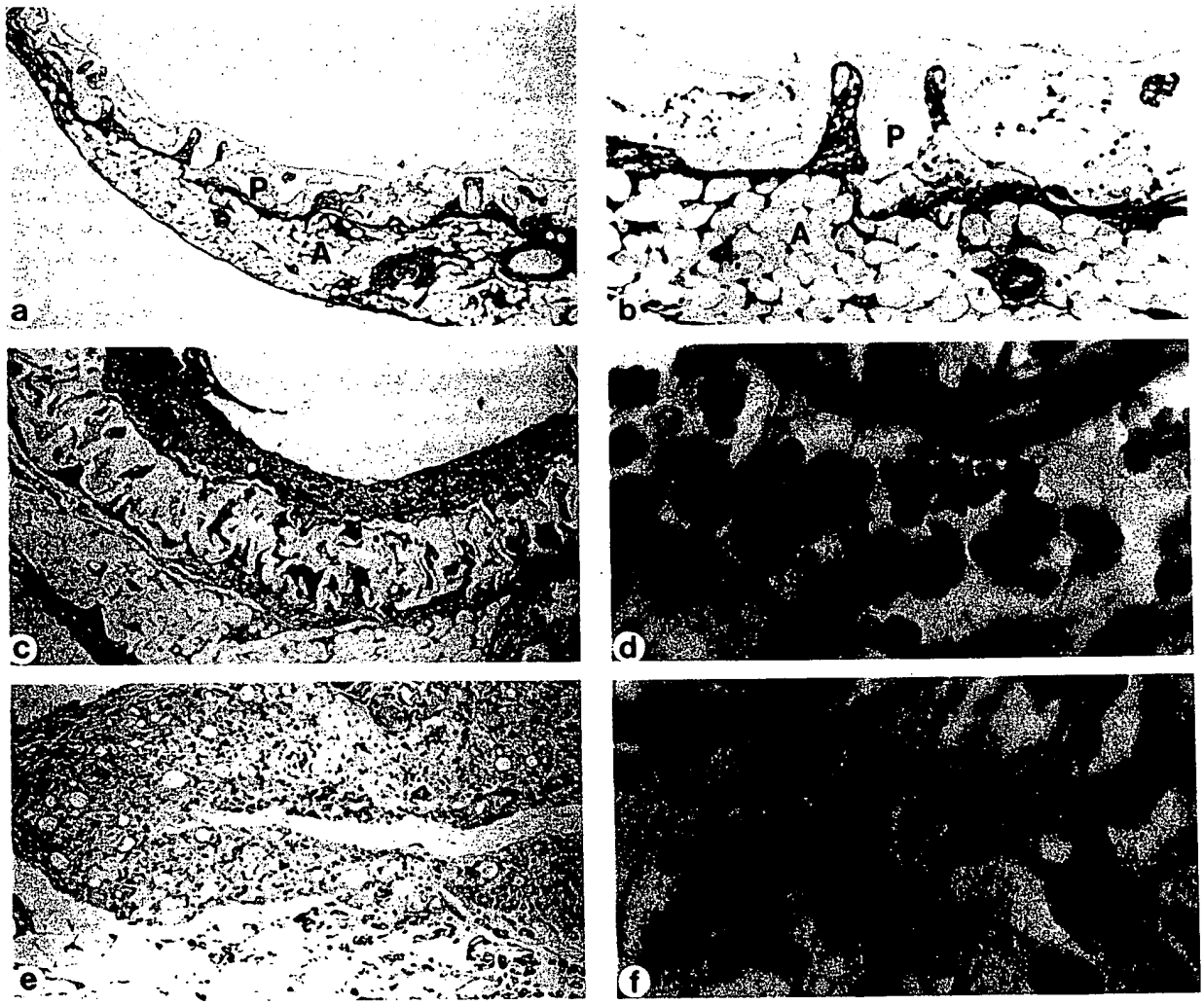

Fig. 4. Tissue ingrowth following device implantation. A low magnification (a), and higher magnification photomicrograph (b) of a device harvested 3 days after implantation. The polymer (P) is surrounded by host tissue, and this consists largely of adipose tissue (A) with occasional, large blood vessels. An inflammatory response to the tissue is visible, and host cells are invading the device via the large pores. (c) Device harvested 7 days after implantation. Fibrovascular tissue has invaded completely through the polymer (P) and formed a layer of granulation tissue on the luminal surface of the device. Multiple blood vessels are apparent in the newly formed tissue. (d) A high magnification of this tissue showing a representative blood vessel. Red blood cells (R) are clearly visible in the new blood vessel. (e and f) A device harvested 56 days after implantation. No evidence of the device was visible in the newly formed tissue under a lower magnification (e), and only under a high magnification (f) were small fragments of the polymer visible (P). Original magnifications were 31× for a, 50× for c, 20× for e, 120× for b, and 1200× for d and f.

ing implantation. To confirm that these devices would degrade rapidly, an in vitro degradation study was carried out. The molecular weight of the devices dropped by approximately 90% within 6 wk, and the mass of the devices subsequently dropped by 90% between wk 6 and 8. The devices had completely degraded by 10 wk (Fig. 3).

Tissue Ingrowth and Vascularization of Devices

Polymer devices were then implanted into the mesenteric tissue of rats to determine both the tissue response to the implants, and the ability of these devices to guide the development of a vascularized, tubular tissue. Immediately following implantation, the host tissue did not interact with the devices (not shown), but by 3 days fibroblasts had begun to infiltrate the pores of the devices (Fig. 4a,b). Seven days after implantation, the invading tissue had grown completely through the devices, and a layer of granulation tissue was formed on the luminal side of the implants (Fig. 4c,d). Vessels containing red blood cells were found throughout the newly formed tissue (Fig. 4d). Examination of this tissue at 56 days indicated that the devices had largely eroded by this point (Fig. 4e), and only under a high magnification could small remnants of the device be visualized (Fig. 4f). The formed tissue remained tubular in structure at this time, with a small central lumen.

Enterocyte Seeding

Finally, to determine the ability of these devices to serve as delivery devices, enterocyte adhesion to these devices in vitro was investigated. Cell suspensions containing from 0.3 to 20 $\times$ 10^6 enterocytes/mL were injected into the lumen of the polymer devices, and enterocyte adhesion was assessed. The number of adherent cells was dependent on the concentration of injected cells, reaching a maximum of approximately 200,000 cells per device (Fig. 5). Scanning electron microscopic examination of cell seeded tubes revealed that this density of cells was essentially a monolayer, while adhesion of lower cell numbers resulted in an incomplete covering of the luminal surface of the devices with enterocytes (Fig. 6).

DISCUSSION

A shortage of donor tissue severely limits the application of organ transplantation and reconstructive surgery to a small percentage of those needing care (1). To alleviate this bottleneck in the source, a variety of groups have proposed engineering new tissue by transplanting not entire tissues, but just the cells of interest (4,11). For selective cell transplantation to become an effective therapy, methods must be developed to both deliver the transplanted cells efficiently to the desired location, and to guide the development of a functioning tissue from the originally disorganized cells.

In this report we describe the design and fabrication of hollow, tubular devices to deliver cells and guide the formation of a vascularized tubular tissue when implanted in vivo. Highly porous films were formed from a biodegradable polyester, and fabricated into hollow tubular devices. Fibrovascular tissue rapidly invaded these devices following implantation into experimental animals, resulting in the formation of a highly vascularized mesenchymal base tissue. The delivery devices, as shown in vitro and in vivo, were sufficiently stable to prevent collapse when placed under a compressive load. The devices were also designed to erode after several months in vivo, and the in vitro and in vivo studies confirmed that the devices were largely degraded by 2 mo.

These results indicate that one can design and fabricate a cell delivery device to promote the formation of a specific tissue. The next step is clearly to utilize these devices to transplant selected cell populations and engineer specific tissue types. The potential of these devices to deliver cells was confirmed by the finding that enterocytes, one cell type of interest in tissue engineering, attached to the devices and formed an organized cell layer on the luminal side of the device. Although a sufficient number of enterocytes attached to form a confluent cell layer, the efficiency of adhesion to these

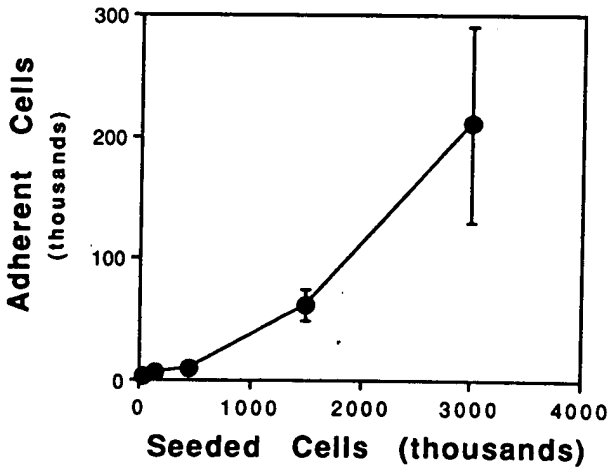

Fig. 5. The density of enterocytes adherent to the device as a function of the density of enterocytes seeded. Enterocytes suspensions were injected into the lumen of devices, and allowed to attach. After 24 h, the caps on the tubes were removed, and adherent cells were removed with trypsin and counted in a Coulter counter. Values represent the mean ± the standard deviation of 4 measurements.

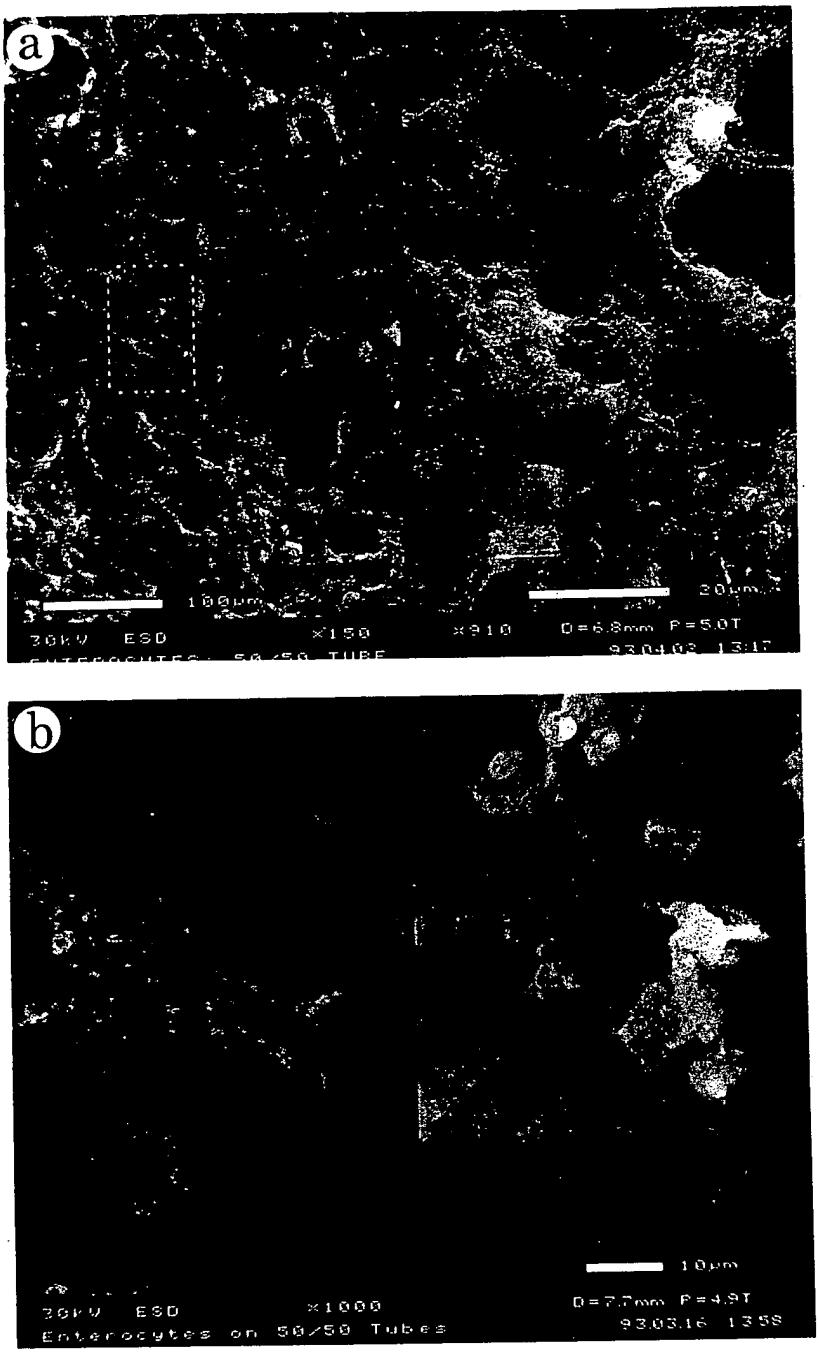

Fig. 6. Scanning electron photomicrographs of devices seeded with (a) 3×10^6 cells/mL, or (b) 0.045×10^6 cells/mL. A lower power and higher power view is shown in each photomicrograph, and the original magnifications are indicated in the photomicrographs.

devices was low, and surface modifications of the device may be needed to improve cell adhesion. Another step would be to transplant several cell types to engineer tissues comprised of multiple cell types. For example, smooth muscle cells could be seeded on the exterior of the delivery devices, and intestinal cells on the interior. An important question in using these devices is when to place the newly engineered tissue online with the existing intestinal tissue. The devices implanted without cells maintained a tubular structure

with a small lumen over the 56 days of the experiment, even though the polymers had largely degraded by that time. Once the newly engineered intestinal tissue has been placed on-line, the transport of materials through the tissue may result in the maintenance of a large lumen, but this issue will need to be investigated.

In conclusion, these results indicate that it is possible to design and fabricate a device to deliver a desired cell type, and guide the development of a desired tissue structure. The specific devices fabricated here may be useful in engineering a variety of tubular tissues, including intestine, blood vessels, and tracheas. Additionally, this approach has allowed us to begin analyzing the assumption that a cell delivery device is capable of controlling the geometry, vascularization, and structure of an engineered tissue.

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REFERENCES

1. Annual report of the U.S. scientific registry for organ transplantation and the organ procurement and transplant network, 1990, UNOS, Richmond, VA, and the Division of organ transplantation, Health Resources and Services Administration, Bethesda, MA, ES9,19.
2. Atala, A.; Vacanti, J.P.; Peters, C.A.; Mandell, J.; Retik, A.B.; Freeman, M.R. Formation of urothelial structures in vivo from disassociated cells attached to biodegradable polymer scaffolds in vitro. J. Urology. 148:658-662; 1992.
3. Dudwick, S.J.; Latif, R.; Fosnocht, D.E. Management of short-bowel syndrome. Surg. Clin. N. Am. 71:625-643; 1991.
4. Langer, R.; Vacanti, J.P. Tissue engineering. Science 260: 920-926; 1993.
5. Mikos, A.G.; Sarakinos, G.; Leite, S.M.; Vacanti, J.P.; Langer, R. Laminated three dimensional biodegradable foams for use in tissue engineering. Biomaterials (in press).
6. Miller, R.A.; Brady, J.M.; Cutright, D.E. Degradation rates of oral resorbable implants (polylactates and polyglycolates): Rate modification with changes in PLA/PGA copolymer ratios. J. Biomed. Mater. Res. 11:711-719; 1977.
7. Mooney, D.J.; Vacanti, J.P. Tissue engineering using cells and synthetic polymers. Transplantation Rev. 7:153-162; 1993.
8. Organ, G.M.; Mooney, D.J.; Hansen, L.K.; Schloo, B.; Vacanti, J.P. Transplantation of enterocytes utilizing polymer-cell constructs to produce neointestine. Transplantation Proc. 24:3009-3011; 1992.
9. Product Information, Medisorb, Cincinnati, Ohio.
10. Reed, A.M.; Gilding, D.K. Biodegradable polymers for use in surgery poly (glycolic)/poly(lactic acid) homo and copolymers: 2. In vitro degradation. Polymer 22:494-498; 1981.
11. Skalak, R.; Fox, C.F. eds.: Tissue engineering, New York: Alan R. Liss; 1988.
12. Vacanti, C.A.; Kim, W.; Upton, J.; Vacanti, M.P.; Mooney, D.; Vacanti, J.P. Tissue engineered growth of bone and cartilage. Transplant Proc. 25:1019-1021; 1993.
13. Vacanti, C.A.; Langer, R.; Schloo, B.; Vacanti, J.P. Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. J. Plastic Recon. Sur. 88(5):753-759; 1991.
14. Vacanti, J.P.; Morse, M.A.; Saltzman, W.M.; Domb, A.J.; Perez-Atayde, A.; Langer, R. Selective cell transplantation using bioabsorbable artificial polymers as matrices. J. Ped. Sur. 23:3-9; 1988.
15. Visscher, G.E.; Robison, R.L.; Maulding, H.V.; Fong, J.W.; Pearson, J.E.; Argentieri, G.J. Biodegradation of and tissue reaction to 50:50 poly (D,L-lactide-*co*-glycolide) microcapsules. J. Biomed. Mater. Res. 19:349-365; 1985.
16. Uyama, S.; Takeda, T.; Vacanti, J.P. Delivery of a whole liver equivalent hepatic mass using polymer devices and hepatotrophic stimulation. Transplantation 55:932-935; 1993.
17. White, R.A.; Hirose, F.M.; Sproat, R.W.; Lawrence, R.S.; Nelson, R.J. Histopathologic observations after short-term implantation of two porous elastomers in dogs. Biomaterials 2:171-176; 1981.

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Human neoplastic submandibular intercalated duct cells express an acinar phenotype when cultured on a basement membrane matrix

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Abstract. Culture of the human neoplastic submandibular gland intercalated duct cell line, HSG, on the basement membrane extract Matrigel induces dramatic morphologic changes and cytodifferentiation. Transmission electron microscopy demonstrated an acinar cell phenotype with polarized cells containing a well-developed Golgi apparatus, multiple microvilli-like projections from the apical surfaces into a lumenal-like area, and numerous granule-like organelles. Amylase, an acinar cell marker, was detected by both immunocytochemical and Northern blot analyses. A 50% reduction in [^{3}H]thymidine incorporation by cells cultured on Matrigel, as compared to cells cultured on tissue culture plates, confirmed the differentiated phenotype of the cells. Multiple components of Matrigel appear to contribute to the morphologic differentiation of the HSG cells since antibodies to both laminin and collagen IV, as well as the laminin-derived bioactive peptide containing SIKVAV, have potent inhibitory effects on HSG cell organization on Matrigel. Collectively, these data indicate that culture of HSG cells on Matrigel is a useful model to study salivary gland acinar development.

Introduction

During development and repair of the major salivary glands, the intercalated duct cell is believed to be the stem cell which gives rise to other ductal, myoepithelial and acinar cell types [5, 13, 29]. The pluripotential nature of the intercalated duct cell has also been suggested in several previous studies using a transformed cell line, HSG, cloned from an irradiated human submandibular gland. Characterization of the HSG cell line, including expression of lactoferrin and secretory components, provided substantial evidence that these cells were derived from the intercalated duct region [33]. In addition, these cells have been shown to have the potential to express a variety of phenotypes when exposed to a number of different exogenous agents in culture. For example, it has been reported that addition of sodium butyrate will induce expression of a myoepithelial phenotype [1]. When HSG cells were cultured in the presence of 5-azacytadine, several stable clones were derived, some developing a myoepithelial phenotype and expressing myosin, while others became acinar-like and expressed amylase, a salivary acinar cell-specific protein [31]. More recently, retinoic acid has been shown to induce HSG cells to adopt a keratinocyte-like phenotype, with well developed cytokeratin filaments [2]. Finally, the stable acinar clone HSG-AZA3, established from the 5-azacytadine-treated cells, was induced to express a chondrocyte-like phenotype by treatment with 1α,25 dihydroxy vitamin D3 [3]. Such studies demonstrate the pluripotent nature of these cells.

While histologic descriptions of salivary gland development outline a sequence of events from the terminal bud stage to the completion of branching morphogenesis with distinct ductal and acinar cell elements [31], mechanisms essential to gland maturation have not yet been elucidated. Early studies of salivary gland development demonstrated that epithelial cell-mesenchymal cell interactions are vital to branching morphogenesis [17]. Subsequent investigations confirmed that the presence of extracellular matrix was essential in establishing and in maintaining the lobular morphologic appearance of developing gland explants [4, 8], and that matrix-epithelial cell-mesenchymal cell interactions were required for stability of the basement membrane in these explants [7, 14, 35].

Extracellular matrices, in particular basement membrane proteins, are key modulators of morphogenesis and differentiation in a variety of specialized epithelial

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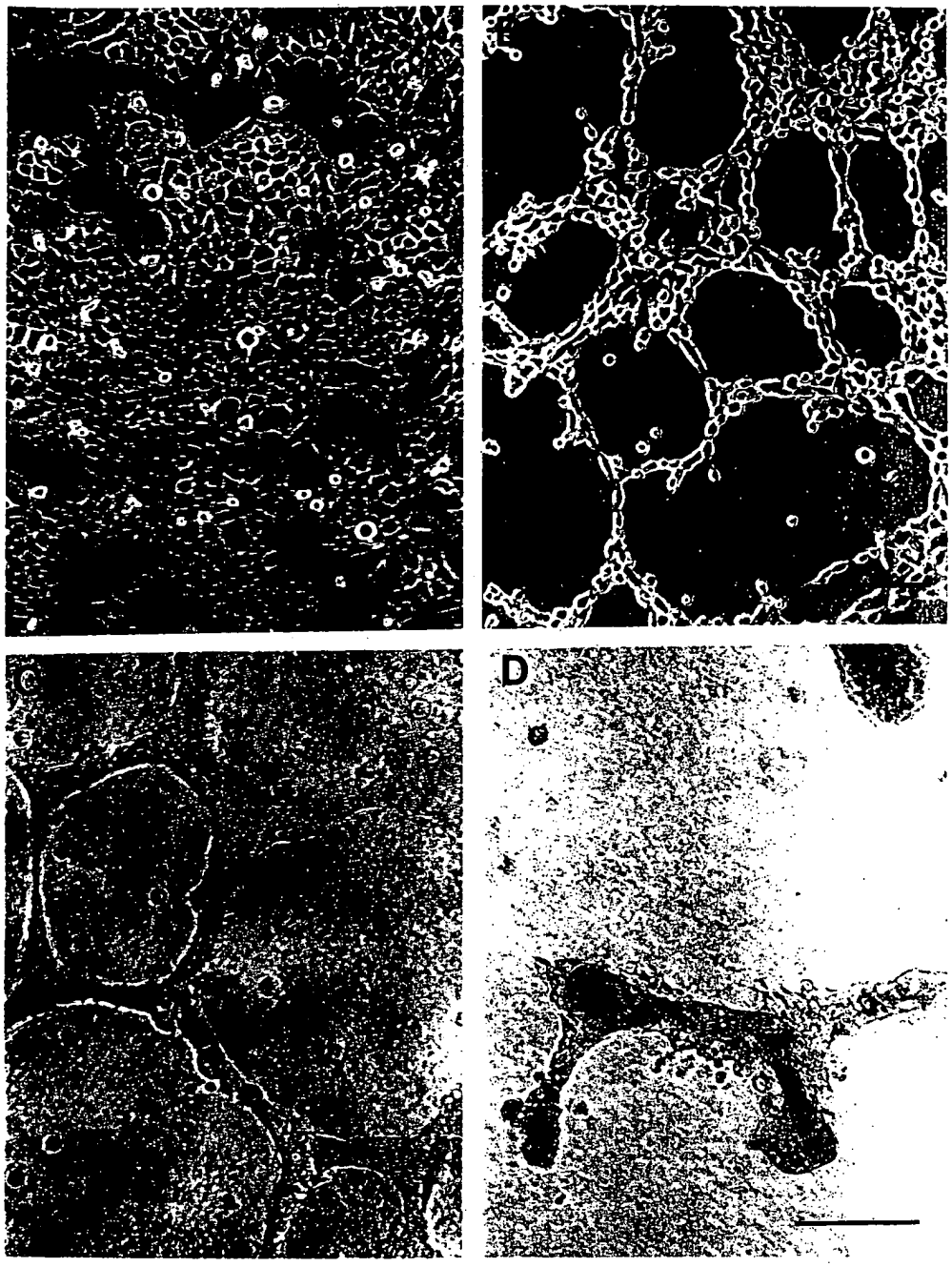

Fig. 1. Phase photomicrographs of HSG cells grown on **A** tissue culture plastic (60 h) or on **B–D** Matrigel for **B** 5 h, **C** 24 h and **D** 60 h. *Bar* **A** and **B**, 200 μm; **C** and **D**, 100 μm

tissues [18, 26]. Epithelial, endothelial and glandular cell differentiation has been observed in vitro with a substratum of reconstituted basement membrane, Matrigel [6, 24, 43]. Matrigel contains primarily laminin as well as collagen type IV, entactin/nidogen, heparan sulfate proteoglycan and a number of growth factors including epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor and transforming growth factor beta [41]. In this study, we have evaluated the effects of in vitro culture of the pluripotent HSG salivary cell line on Matrigel.

Methods

Cell Culture. The neoplastic HSG cell line, obtained as a gift from Dr. Mitsunobu Sato, Tokushima University, Japan, was maintained in a complete medium of Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 1:1 (Biofluids, Rockville, Md., USA) containing 10% fetal bovine serum (Biofluids), 292 μg/ml glutamine, and 100 U/ml penicillin-100 μg/ml streptomycin (Gibco, Grand Island, N.Y., USA). The cells were maintained at 37° C in a humidified 5% CO_2/95% air atmosphere.

Matrigel, collagen I, collagen IV and laminin culture assay. Matrigel, laminin and collagen IV were prepared as previously described [21, 22, 40]. Matrigel was diluted to 10 mg/ml with media, rat tail collagen I (Sigma, St. Louis, Mo., USA) diluted to 2 mg/ml in 0.5 *M* acetic acid, collagen IV diluted to 0.8 mg/ml in 0.5 *M* acetic acid, or laminin diluted to 2 mg ml in media, was coated on the bottom of a 24 well (250 ul/16 mm well) tissue cultured plate (Falcon, Cockeysville, Md., USA), and then incubated at 37° C for 30 min. Complete medium (500 μl) was gently pipetted onto the Matrigel layer, followed by 8×10^4 cells, which had been released from culture dishes with a buffered 0.05% trypsin/

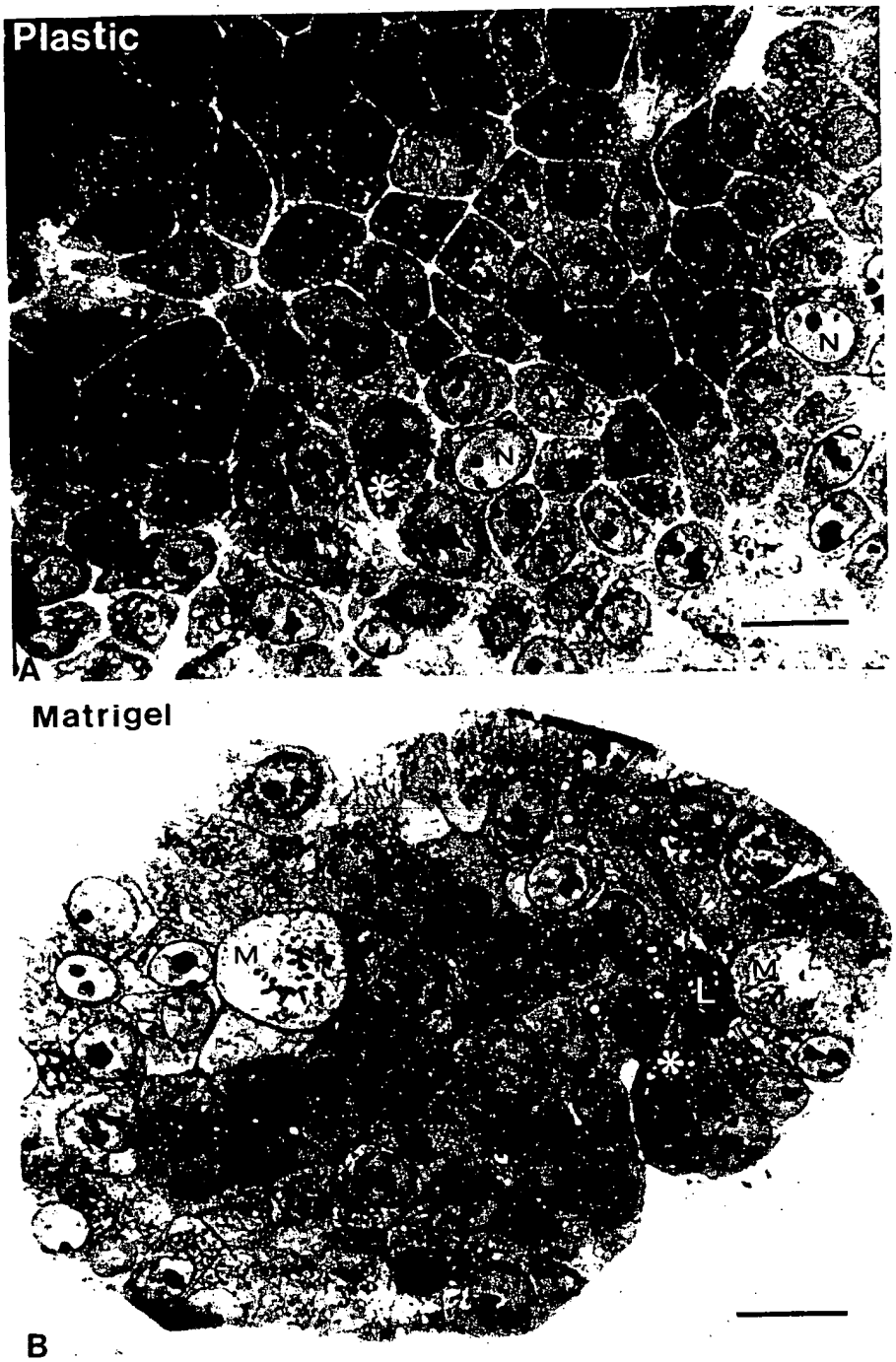

Fig. 2. Light photomicrographs of 1 μm thick sections of HSG cells grown **A** on tissue culture plastic, or **B** on Matrigel. On the plastic (**A**), the cells formed a monolayer and generally appeared undifferentiated. The spherical nuclei (*N*) were surrounded by cytoplasm (*asterisk*). On Matrigel (**B**), the cells organized into rounded acinar-like structures. The nuclei were often found in a basal position with abundant supranuclear cytoplasm (*asterisk*). Cells often assumed a columnar shape and appeared to organize around a lumen-like structure. Note the group of cells at the right of the photograph. Darkly-stained material was noted in the presumptive lumen (*L*). Dividing cells (*M*) were noted both on plastic and on Matrigel. Both of these sections were cut parallel to the bottom of the dish. However, since the acinar-like structures resembled true acini, sections perpendicular or oblique to the section noted in **B** looked generally similar. *Bars*, 50 μm

0.53 m*M* EDTA solution (Gibco) and resuspended at 1×10^6 cells/ml in complete medium. Parallel cultures on tissue culture plates were simultaneously prepared. The cells were photographed daily using phase photomicroscopy. At 72 h, cells from all culture conditions were fixed in 5% glutaraldehyde/0.2 *M* collidine buffer, post-fixed in a 1:1 mixture of 3% potassium ferrocyanide and 2% osmium tetroxide, and Epon embedded. Thick sections of 1 μm were prepared and stained with toluidine blue. Subsequently, thin sections were prepared and were photographed by transmission electron microscopy using a JEOL 1200-EX.

Immunocytochemistry. Matrigel cultures were harvested, embedded with Histo Prep Frozen Tissue Embedding Media (Fisher Scientific, Orangeburg, N.Y., USA), frozen in liquid nitrogen and stored at −70° C prior to sectioning. Cryostat sections were fixed in 4% formaldehyde at room temperature for 15 min, immersed in phosphate buffered saline (PBS) containing 0.6% H_2O_2 to inactivate endogenous peroxidase, and then the sections were blocked with non-immunized goat serum (Zymed Laboratory, San Francisco, Calif., USA). Human parotid gland frozen sections were used as a positive control. HSG cells, seeded onto 8-well glass chamber slides (Nunc, Naperville, Ill., USA) and cultured for 72 h, were fixed with absolute methanol at −20° C for 15 min, immersed in 0.06% H_2O_2, and blocked with non-immunized goat serum. Either rabbit antisera to human salivary amylase [kindly provided by Dr. Alfredo Aguirre (SUNY at Buffalo, N.Y., USA)], or normal rabbit serum (Gibco), were used as the primary antibody at a dilution of 1:100 and developed as per the Histostain-Sp Kit (Zymed Laboratory).

Northern blot analysis. Parallel cell cultures on tissue culture dishes and on Matrigel were harvested at 48 h for extraction and isolation

of RNA using the guanidine thiocyanate method [10]. Total RNA was quantitated and stored in ethanol at −20° C. Twenty micrograms of RNA was electrophoresed in a 1.2% formaldehyde agarose gel, blotted onto a Nytran membrane (Schleicher and Schuell, Keene, N.H., USA), cross-linked to the membrane by baking under vacuum, prehybridized and then hybridized with [α-^{32}P]dCTP-labeled human salivary amylase cDNA (ATCC, Rockville, Md., USA) labeled by the random primer method (specific activity of greater than 10^6 dpm/μg) using the Random-Primer DNA Labeling System kit (BRL, Gaithersburg, Md., USA). Hybridization with a ^{32}P-labeled 18S genomic DNA fragment encoding *Schistosoma mansoni* 18S ribosomal RNA was used to evaluate loading and transfer of RNA in the Northern blot assays. Densitometry was performed to normalize hybridized samples.

[^{3}H]Thymidine incorporation. Parallel cell cultures on Matrigel and on tissue culture dishes in complete media were labeled with 0.5 μCi of [^{3}H]thymidine for one hour at 24 h intervals. After labeling, the media were aspirated. The cells were washed three times with PBS, and exposed to Dispase (Collaborative Research, Bedford, Mass., USA) for 30 min at 37° C. The contents of the well were thus released and pelleted. The cell pellets were resuspended in 15% trichloroacetic acid (TCA) and allowed to precipitate overnight at 4° C. After centrifugation, the pellets were washed with 5% TCA, centrifuged again, and resuspended in 0.1 N NaOH. Aliquots were added to Aquasol scintillant (Dupont, Boston, Mass., USA) and counted in a Beckman LS 3801 scintillation counter. Each time point was performed in triplicate and the experiment was repeated three times.

Antibody inhibition assay. Antibodies to human placental laminin (Calbiochem, La Jolla, Calif., USA) and to human type IV collagen (Chemicon, Temecular, Calif., USA) as well as normal rabbit serum were heat inactivated at 56° C for 30 min. Each antibody was then added in duplicate to Matrigel-coated wells at a 1:10, 1:20 or 1:40 final dilution in 100 μl of complete media containing heat inactivated serum. The culture plates were gently rocked for several minutes to allow for coating of the Matrigel surface. Then 8×10^4 cells in 80 μl of complete media were pipetted into each well and the total media volume was brought to 500 μl. The cultures were maintained for a period of three days. The cells were fixed in cold absolute methanol, stained with hematoxylin and eosin, and photographed.

Peptide inhibition assay. Synthetic peptides corresponding to laminin sequences, CSRARKQAASIKVAVSADR (residues 2091–2109) from the A chain, a YIGSR trimer (CYIGSRYIGSRYIGSR; residues 929–933) from the short arm of the B1 chain, and a control inactive laminin-derived peptide, Y6, (residues 615–634) also from the short arm of the B1 chain were synthesized in the amide form and HPLC-analyzed as previously described [15]. The peptides were solubilized in DMEM and added in duplicate to Matrigel-coated wells at a concentration of 0.1 μg, 10.0 μg and 100.0 μg/well. Complete media (100 μl) were pipetted onto the wells and the culture plates were gently rocked for several minutes in order to evenly coat the Matrigel surface. To each well 8×10^4 cells suspended in complete media were added and the total media volume was brought to 500 μl. The cultures were observed daily, maintained for three days, and then fixed in cold absolute methanol, stained with hematoxylin and eosin and photographed.

Results

The morphology of HSG cells on tissue culture plates and on Matrigel was examined. On plastic, these cells formed islands or clusters which coalesced as confluence was achieved (Fig. 1 A). One micron thick sections of these cells revealed a monolayer of cells with a relatively undifferentiated appearance, consisting of an often centrally located nucleus with cell organelles dispersed in the cytoplasm (Fig. 2 A). At times, large areas of the cytoplasm were devoid of organelles. Transmission electron microscopic (TEM) analysis revealed the cell organ-

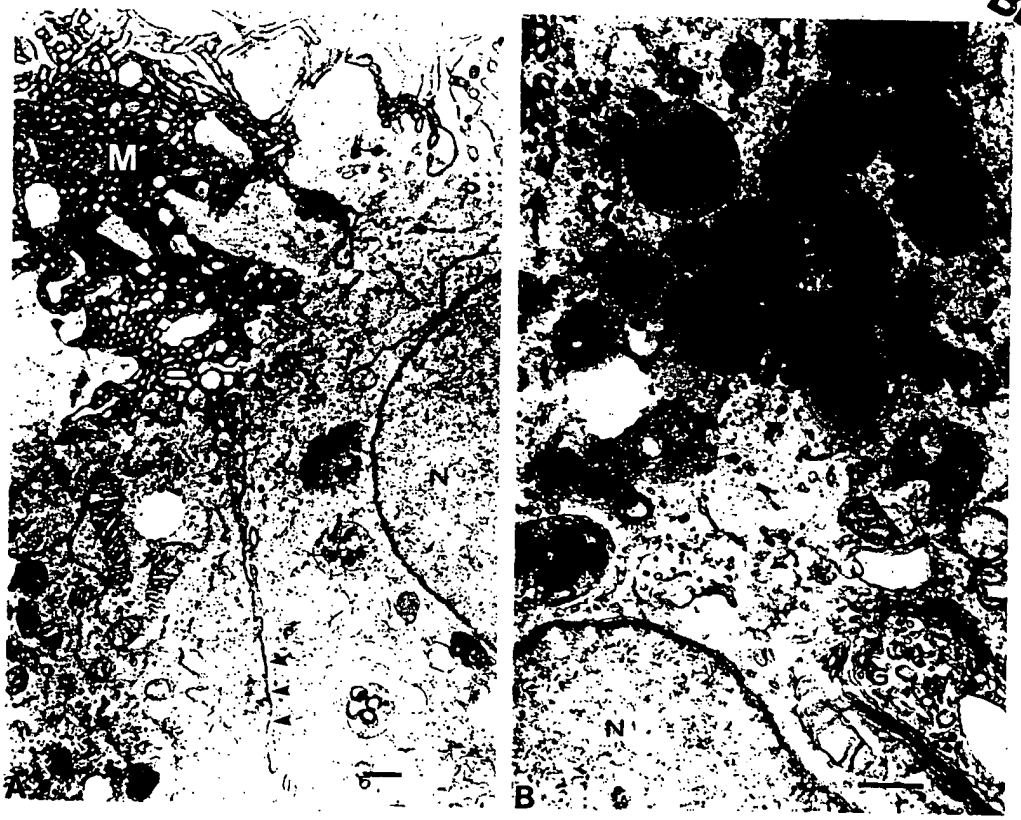

Fig. 3. Transmission electron micrographs of HSG cells grown on Matrigel. The presumptive lumenal area (see Fig. 2 B) is shown in the left panel (A). The apical surface of two cells are noted. Numerous finger-like, interdigitating plasma membrane projections (*M*) are seen in the lumen. The density inside the projections appears very similar to microvilli projections, although in this instance they are far more irregular than typical microvilli. *Arrowheads* point to the plasma membranes of the cells. The cell on the right shows abundant granule-like organelles (*D*) and a Golgi (*G*) apparatus adjacent to the nucleus (*N*). *Bars*, 2 μm

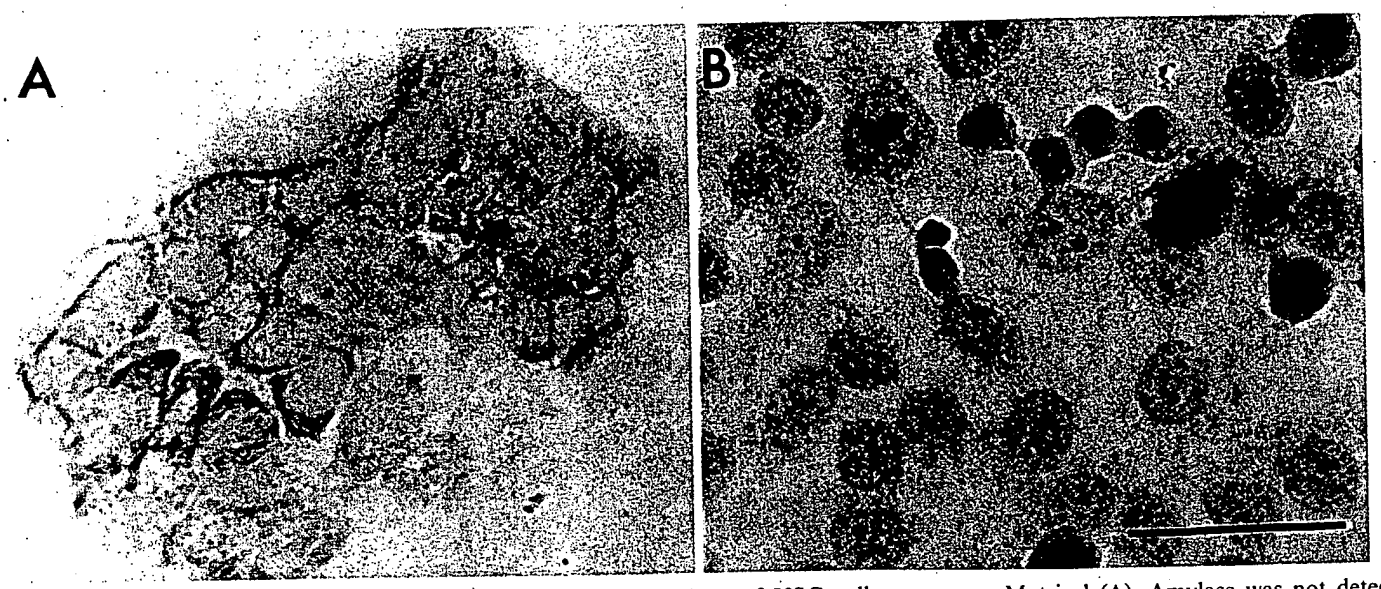

Fig. 4. Immunocytochemical detection of amylase in frozen sections of HSG cells grown on Matrigel (**A**). Amylase was not detected in HSG cells grown on glass slides (**B**). *Bar*, 50 μm

elles to be primarily mitochondria, with no secretory granules or granule-like organelles present (data not shown).

When cultured on Matrigel, however, these cells exhibit a dramatic change in morphologic appearance, rapidly aligning into a reticular network which, over the course of three days, breaks apart, often resulting in individual three dimensional cords with blunted, tight, multicellular termini (Fig. 1 B–D). One micron thick sections of cells cultured on Matrigel for 72 h revealed these cells to be organized into acinar-like structures, with basally-displaced nuclei and abundant supranuclear cytoplasm (Fig. 2 B). Transmission electron microscopic analysis revealed multiple microvilli-like structures at the apical surfaces projecting into a presumptive lumenal area (Fig. 3 A). In addition, there is the novel appearance of numerous granule-like organelles (Fig. 3 B). This phenotype, which is induced in response to Matrigel is indicative of a cell specialized for secretion.

Frozen sections were immunostained for the presence of amylase to determine if an induction of salivary-specific proteins accompanied the morphologic change observed in cells cultured on Matrigel. Antisera to human salivary amylase detected the exocrine secretory protein in the cytoplasm of cells cultured on Matrigel (Fig. 4 A), but not in cells cultured on glass slides (Fig. 4 B). As well, normal rabbit serum applied in place of a primary antibody to sections of cells cultured on Matrigel was not immunoreactive. Total RNA was harvested from cells cultured either on Matrigel or tissue culture plates at 48 h and was probed for the presence of amylase message. An approximate 20-fold increase in induction of message for amylase was detected when cells were cultured on Matrigel (Fig. 5 A).

Over a 72 h time period, the cells grown on plastic approached confluence, whereas the cells cultured on Matrigel grew considerably slower. [^{3}H]Thymidine incorporation by cells on Matrigel was less than 50% of that incorporated by cells cultured on plastic for each

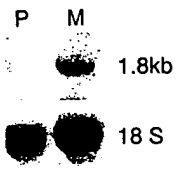

Fig. 5. Northern blot detection of 1.8 kb amylase message in total RNA harvested from cells cultured on tissue culture plastic (*P*) or from cells cultured on Matrigel (*M*) (*upper panel*). For densitometric quantitation, the loading of total RNA of each sample was normalized by hybridization with a ^{32}P-labeled probe for 18S ribosomal RNA (*lower panel*)

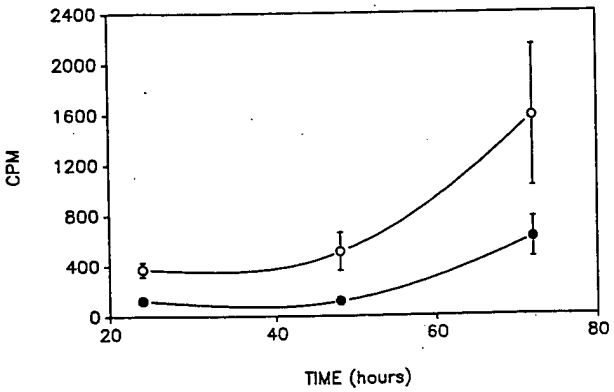

Fig. 6. [^{3}H]Thymidine incorporation by cells cultured on tissue culture plastic (*open circles*) or by cells cultured on Matrigel (closed circles). Time points included 24 h, 48 h and 72 h ($n=3$ for all time points, except tissue culture plastic at 12 h where $n=6$). The mean +/− standard deviations are plotted (except for cells on Matrigel at 24 and 48 h, where numerical values were too small to be plotted)

time point tested, indicating a lengthened cell cycle (Fig. 6).

As Matrigel is a mixture of several proteins, we attempted to discern the role of the two major constituents (laminin and collagen IV) in the differentiaton process. First, HSG cells were cultured on a layer of laminin

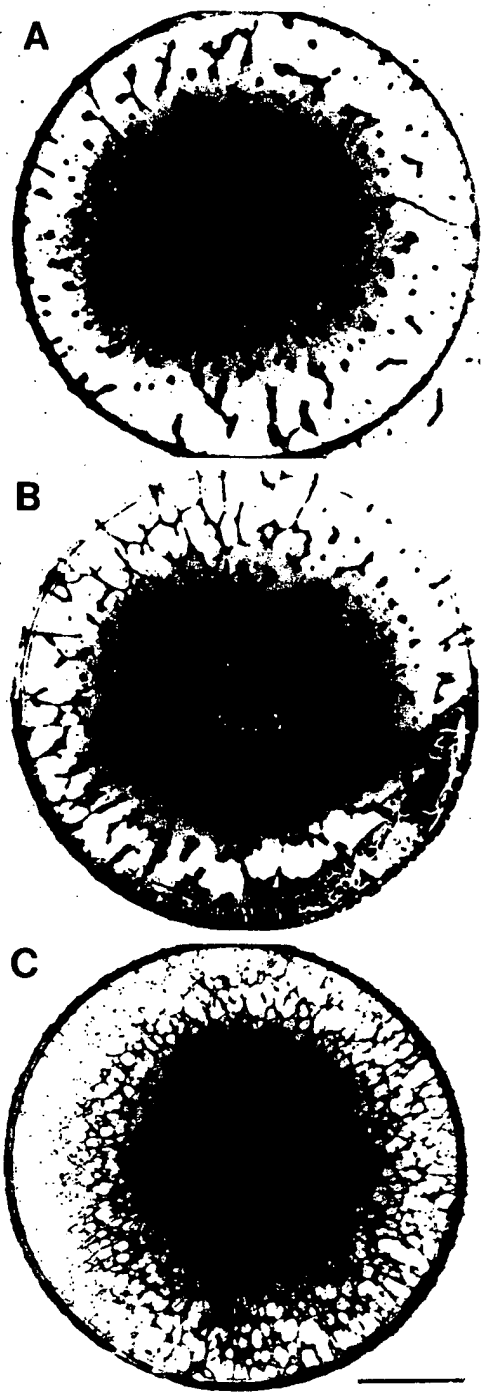

Fig. 7. Antibody inhibition assay showing representative 16 mm wells of HSG cells grown on Matrigel in the presence of a 1:20 dilution of **A** normal rabbit serum, **B** antibody to human laminin, or **C** antibody to human collagen type IV. *Bar*, 4 mm

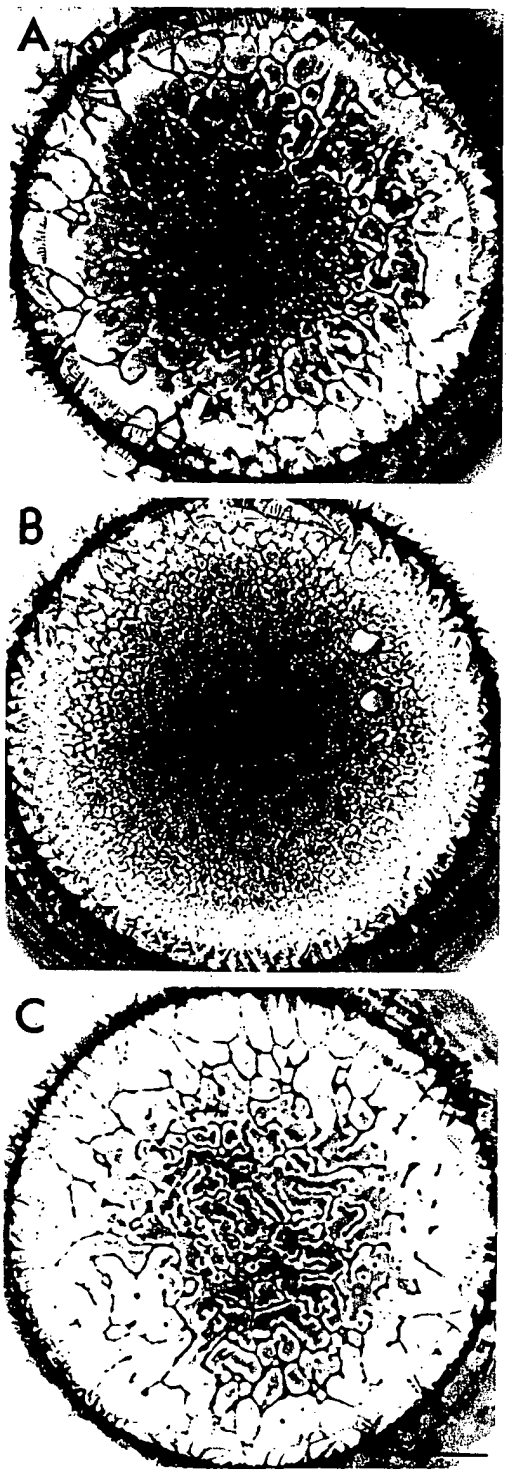

Fig. 8. Laminin-derived peptide inhibition assay showing dose response of HSG cells grown on Matrigel in the presence of **A** 10 μg of SIKVAV peptide, **B** 100 μg of SIKVAV peptide and **C** 100 μg of the control peptide, Y6. The response of the cells to 0.1 μg of SIKVAV was equivalent to that seen in **C**. *Bar*, 4 mm

in an effort to determine if the morphologic change/cytodifferentiation was primarily in response to this protein. While HSG cells cultured on laminin alone underwent a morphologic change similar to that seen on Matrigel, electron microscopic analysis revealed that the cells were not polarized and looked similar to those cultured on tissue culture plastic, with the exception that cells cultured on laminin did have microvilli such as those seen in cells cultured on Matrigel (data not shown). HSG cells cultured on either collagen I or on collagen IV expressed a phenotype similar to that seen on tissue culture plastic (data not shown). Next, antibodies to laminin and to collagen type IV were used to examine further the role of these proteins in the morphogenetic changes. Antibodies to both proteins inhibited the organization of HSG cells on Matrigel (Fig. 7). While the cells did align into the reticular network, coculture of

cells with laminin antibodies resulted in formation of thicker cord-like structures which did not separate (Fig. 7B). In the presence of collagen IV antibodies, the cells formed a fine reticular network which was distinct from the response to Matrigel alone (Fig. 7C). Examination at a higher power revealed that the cells in the network patches were flat and had a monolayer appearance (not shown). The effect of the antibodies was sustained for the three day duration of the experiment and was most potent at a 1:10 dilution for the laminin antisera relative to normal rabbit serum. The effect was also evident at a 1:20 dilution. A 1:10 dilution of the collagen type IV antibody did not allow cell attachment (data not shown). This further indicates the importance of type IV collagen in cell attachment, since the control sera and the laminin antibodies did not have this effect.

Since several active domains of laminin have been defined by synthetic peptides [15, 23, 38, 39], we assessed whether such peptides could influence the morphologic differentiation of HSG cells on Matrigel. A synthetic peptide corresponding to a site on the A chain of laminin containing the SIKVAV sequence, did show a dose-dependent inhibition of the organization of HSG cells on Matrigel (Fig. 8). Other laminin derived peptides tested, including the bioactive YIGSR and inactive Y6, essentially had no inhibitory effect (Fig. 8C). We conclude that both laminin and collagen IV are required for the cytodifferentiation of these cells.

Discussion

Here we have demonstrated that pluripotent, neoplastic HSG intercalated duct cells, which have previously been shown to express a variety of phenotypes [1, 2, 3, 32], can be induced to express an acinar phenotype when cultured on basement membrane Matrigel. Transmission electron microscopic analysis of cells cultured for 72 h on Matrigel revealed cells tightly bound to each other, with secretory cell characteristics, including apical surfaces lined with microvilli-like projections, a well-developed Golgi and granule-like organelles. [^{3}H]Thymidine incorporation by cells cultured on Matrigel was approximately half that of cells cultured on plastic and was similar to the doubling time of HSG-AZA3 cells, an acinar-like stable clone which also expresses amylase [32]. Furthermore, amylase, an exocrine secretory protein considered a marker for acinar cells, was detected by both Northern blot and immunocytochemistry. These data are consistent with the intercalated duct cell-like characteristics of the HSG cells and with the hypothesis that the intercalated duct cell is a salivary stem cell.

While early investigations into salivary gland development established that mesenchymal cell-epithelial cell-matrix interactions are important for stability of the lobular morphologic appearance [4, 7, 35], the mechanisms for this differentiation remain unclear. Here we have demonstrated that basement membrane proteins are key modulators of morphogenetic change and of cytodifferentiation of the HSG intercalated duct cells. Matrigel has been used in many laboratories as a means of preserving, enhancing or inducing the differentiated phenotype of a variety of epithelial cells, including rat salivary submandibular rudiments [18, 26, 37]. Recently, the control of mammary tissue-specific gene expression of β-casein by mouse mammary epithelial cells in vitro has been linked specifically to contact of these cells with reconstituted basement membrane in culture, in the absence of cell-cell contact or mesenchymal cues [36].

We have also determined through antibody inhibition assays, that at least laminin and collagen type IV are necessary for the organization of HSG cells on Matrigel. These data are consistent with those of Cutler, who reported that addition of laminin antibodies to 16 day embryonic rat submandibular gland rudiments in culture reduced levels of cytodifferentiation, while addition of collagen IV antibodies essentially blocked morphogenesis and cytodifferentiation [11].

The culture of differentiated rodent and murine salivary acinar cells has been intensely pursued in several laboratories [12, 28, 30], and Matrigel has been a substratum of choice in maintaining the differentiated phenotype in several of these model systems. These cultures are generally short-lived and require dissection and dissociation of explants. Here, we demonstrate induction of cytodifferentiation of an undifferentiated human neoplastic salivary duct cell to an acinar phenotype, complete with the cells related in an acinar-like structure. Although this system utilizes a transformed duct cell line as opposed to cells isolated in a differentiated state, it offers a predictable and readily available model to further study salivary gland tissue-specific gene expression mediated by extracellular matrix proteins. Since acinar maturation coincides with innervation in the developing rat [31], it may be that enhanced expression of amylase or induction of other salivary specific proteins such as proline-rich proteins or submandibular-specific cystatin, can be accomplished through addition of neurotransmitters or hormones. Other soluble proteins, such as growth factors, could also play an important role in morphogenetic events. For example, epidermal growth factor (EGF) (which is in Matrigel) has recently been reported as an epigenetic factor in lung branching morphogenesis [42]. and was found to enhance branching morphogenesis in salivary gland rudiments cultured in Matrigel [27].

While culture of HSG cells on laminin alone induced organization of HSG cells in a macroscopic manner similar to that of HSG cells cultured on Matrigel, TEM analysis did not indicate an induction of cytodifferentiation by laminin. Since the SIKVAV site appears to be active in blocking HSG cell organization on Matrigel, a role for laminin is clear. Thus, these data demonstrate that laminin has a role in cytodifferentiation but that laminin alone is not sufficient. It is interesting to note that a YIGSR-containing peptide derived from laminin was inactive with HSG cells. Previously, we found that a submandibular tumor cell line A253 differentiated on Matrigel and this response was blocked by SIKVAV containing peptides, but not by YIGSR [20]. In contrast, YIGSR-containing peptides were previously shown to inhibit endothelial cell differentiation and outgrowth

from the neural crest when plated on Matrigel [9, 16]. In fact, endothelial cells have been shown to recognize at least two sites (YIGSR and RGD) on laminin, and peptides comprising both sites block differentiation into capillary-like structures. The present study therefore describes a unique specificity, to date, of the HSG cells in their interaction with laminin. Since laminin has at least seven active sites described by synthetic peptides [15, 23, 34, 38, 39] and others are likely to exist, it is possible that HSG cells recognize additional sequences in laminin. It should also be noted that isoforms of laminin have recently been described [19, 25] and their role in the developing salivary gland is yet to be determined.

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References

1. Azuma M, Hayashi Y, Yoshida H, Yanagawa T, Yura Y, Ueno A, Sato M (1986) Emergence of differentiated subclones from a human salivary adenocarcinoma cell clone in culture after treatment with sodium butyrate. Cancer Res 46:770–777
2. Azuma M, Kawamata H, Kasai Y, Nagamine S, Yoshida H, Yanagawa T, Sato M (1988) Effects of retinoic acid on morphologic features and biologic markers of a neoplastic human salivary intercalated duct cell line in culture. Cancer Res 48:7219–7225
3. Azuma M, Kawamata H, Kasai Y, Yanagawa T, Yoshida H, Sato M (1989) Induction of cells with a chondrocyte-like phenotype by treatment with 1α,25-dihydroxyvitamin D_3 in a human salivary acinar cell line. Cancer Res 49:5435–5442
4. Banerjee SD, Cohn RH, Bernfield MR (1977) Basal lamina of embryonic salivary epithelia. Production by the epithelium and role in maintaining lobular morphology. J Cell Biol 73:445–463
5. Batsakis JG (1980) Salivary gland neoplasia: an outcome of modified morphogenesis and cytodifferentiation. Oral Surg 49:229–232
6. Bendayan M, Duhr M, Gingras D (1986) Studies on pancreatic acinar cells in tissue culture: basal lamina (basement membrane) matrix promotes three-dimensional reorganization. Eur J Cell Biol 42:60–67
7. Bernfield MR, Banerjee SD (1982) The turnover of basal lamina glycosaminoglycan correlates with epithelial morphogenesis. Dev Biol 90:291–305
8. Bernfield MR, Banerjee SD, Cohn RH (1972) Dependence of salivary epithelial morphology and branching morphogenesis upon acid mucopolysaccharide-protein (proteoglycan) at the epithelial surface. J Cell Biol 52:674–689
9. Bilozur ME, Hay ED (1988) Neural crest migration in 3D extracellular matrix utilizes laminin, fibronectin and collagen. Dev Biol 125:19–33
10. Chirgwin JJ, Przbyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299
11. Cutler LS (1990) The role of extracellular matrix in the morphogenesis and differentiation of salivary glands. Adv Dent Res 4:27–33
12. Durban EM (1990) Mouse submandibular salivary epithelial cell growth and differentiation in long-term culture: influence of the extracellular matrix. In Vitro Cell Dev Biol 26:33–43
13. Eversole LR (1971) Histogenic classification of salivary tumors. Arch Pathol 92:433–443
14. Fukuda Y, Masuda Y, Kishi J, Hashimoto Y, Hayakawa T, Nogawa H, Nakanishi Y (1988) The role of interstitial collagens in cleft formation of mouse embryonic submandibular gland during initial branching. Development 103:259–267
15. Graf J, Iwamoto Y, Sasaki M, Martin GR, Kleinman HK, Robey FA, Yamada Y (1987) Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis and receptor binding. Cell 48:989–996
16. Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin GR, Kleinman HK (1989) Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell 58:933–943
17. Grobstein C (1953) Epithelio-mesenchymal specificity in the morphogenesis of mouse sub-mandibular rudiments in vitro. J Exp Zool 124:383–413
18. Hadley MA, Byers SW, Suarez-Quian CA, Kleinman HK, Dym M (1985) Extracellular matrix regulates sertoli cell differentiation, testicular cord formation, and germ cell differentiation. J Cell Biol 101:1511–1522
19. Hunter DD, Shah V, Merlie JP, Sanes JR (1989) A laminin-like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. Nature 338:229–234
20. Kibbey MC, Royce LS, Dym M, Baum BJ, Kleinman HK (1992) Glandular-like morphogenesis of the human submandibular tumor cell line A253 on basement membrane components. Exp Cell Res 198:343–351
21. Kleinman HK, McGarvey ML, Liotta LA, Gehron Robey P, Tryggvason K, Martin GR (1982) Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma, Biochemistry 21:6188–6193
22. Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR (1986) Basement membrane complexes with biological activity. Biochemistry 25:312–318
23. Kleinman HK, Graf J, Iwamoto Y, Sasaki M, Schasteen CS, Yamada Y, Martin GR, Robey FA (1989) Identification of a second active site in laminin for promotion of cell adhesion and migration and inhibition of in vivo melanoma lung colonization. Arch Biochem Biophys 272:39–45
24. Kubota Y, Kleinman HK, Martin GR, Lawley TJ (1988) Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol 107:1589–1598
25. Leivo I, Engvall E (1988) Merosin, a protein specific for basement membranes of Schwann cells, striated muscle and trophoblast, is expressed late in nerve and muscle development. Proc Natl Acad Sci USA 85:1544–1548
26. Li LM, Aggeler J, Farson DA, Hatier C, Hassell J, Bissell MJ (1987) Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cell. Proc Natl Acad Sci USA 84:136–140
27. Nogawa H, Takahashi Y (1991) Substitution for mesenchyme by basement-membrane-like substratum and epidermal growth factor in inducing branching morphogenesis of mouse salivary epithelium. Development 112:855–861
28. Oliver C, Waters JF, Tolbert CL, Kleinman HK (1987) Growth of exocrine acinar cells on a reconstituted basement membrane gel. In Vitro Cell Dev Biol 23:465–473
29. Pierce GB (1974) Neoplasms, differentiation and mutation. Am J Pathol 77:103–118
30. Quissell DO, Redman RS, Mark MR (1986) Short-term primary culture of acinar-intercalated duct complexes from rat submandibular glands. In Vitro Cell Dev Biol 22:469–480
31. Redman RS (1987) Development of the salivary glands. In: Sreebny LM (ed) The salivary system, Vol. 1. Boca Raton, Florida, pp 1–20
32. Sato M, Azuma M, Hayashi Y, Yoshida H, Yanagawa T, Yura Y (1987) 5-Azacytidine induction of stable myoepithelial and acinar cells from a human salivary intercalated duct cell clone. Cancer Res 47:4453–4459

33. Shirasuna K, Sato M, Miyazaki T (1981) A neoplastic epithelial duct cell line established from an irradiated human salivary gland. Cancer Res 48:745–752
34. Skubitz APN, Letourneau PC, Wayner E, Furcht LT (1991) Synthetic peptides from the carboxyl-terminal globular domain of the A chain of laminin: their ability to promote cell adhesion and neurite outgrowth, and interact with heparin and the $\beta 1$ integrin subunit. J Cell Biol 115:1137–1148
35. Spooner BS, Faubion JM (1980) Collagen involvement in branching morphogenesis of embryonic lung and salivary gland. Dev Biol 77:84-102
36. Streuli CH, Bailey N, Bissell MJ (1991) Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. J Cell Biol 115:1383–1395
37. Takahashi Y, Nogawa H (1991) Branching morphogenesis of mouse salivary epithelium in basement membrane-like substratum separated from mesenchyme by the membrane filter. Development 111:327–335
38. Tashiro K, Sephel GC, Weeks B, Sasaki M, Martin GR, Kleinman HK, Yamada Y (1989) A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration, and neurite outgrowth. J Biol Chem 264:16174–16182
39. Tashiro K, Sephel GC, Greatorex D, Sasaki M, Shiraishi N, Martin GR, Kleinman HK, Yamada Y (1991) The RGD containing site of the mouse laminin A chain is active for cell attachment, spreading, migration and neurite outgrowth. J Cell Physiol 46:451–459
40. Timpl R, Rhode H, Gehron-Robey P, Rennard SI, Foidart JM, Martin GR (1979) Laminin – A glycoprotein from basement membranes. J Biol Chem 254:9933–9937
41. Vukicevic S, Kleinman HK, Luyten FP, Roberts AB, Roche NS, Reddi AH (1992) Identification of multiple active growth factors in basement membrane matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. Exp Cell Res 202:1–8
42. Warburton D, Seth R, Shum L, Horcher PC, Hall FL, Werb Z, Slavkin HC (1992) Epigenetic role of epidermal growth factor expression and signalling in embryonic mouse lung morphogenesis. Dev Biol 149:123–133
43. White TEK, DiSant'Agnese PA, Miller RK (1990) Human endometrial cells grown on an extracellular matrix form simple columnar epithelial and glands. In Vitro Cell Dev Biol 26:636–642

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Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands

(gene transfer/adenovirus/radiation damage/xerostomia)

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ABSTRACT **A replication-deficient, recombinant adenovirus encoding human aquaporin-1 (hAQP1), the archetypal water channel, was constructed. This virus, AdhAQP1, directed hAQP1 expression in several epithelial cell lines *in vitro*. In polarized MDCK cell monolayers, hAQP1 was localized in the apical and basolateral plasma membranes. Fluid movement across monolayers infected by AdhAQP1 in response to an osmotic gradient was ≈4-fold that seen with uninfected monolayers or monolayers infected by a control virus. When AdhAQP1 was administered to rat submandibular glands by retrograde ductal instillation, significant hAQP1 expression was observed by Western blot analysis in crude plasma membranes and by immunohistochemical staining in both acinar and ductal cells. Three or four months after exposure to a single radiation dose (17.5 or 21 Gy, respectively), AdhAQP1 administration to rat submandibular glands led to a two- to threefold increase in salivary secretion compared with secretion from glands administered a control virus. These results suggest that hAQP1 gene transfer may have potential as an unique approach for the treatment of postradiation salivary hypofunction.**

Each year in the United States ≈40,000 new cases of head and neck cancer are diagnosed (1). For most of these patients, ionizing radiation is a key component of therapy. However, such treatment often results in severe damage to the fluid-secreting portion (acinar cells) of the salivary glands that lie in the radiation field (2–5). Patients with reduced salivary secretion suffer from dysphagia, xerostomia, mucositis, dental caries, and frequent oropharyngeal infections. Their quality of life is significantly diminished. Despite recognition and study of these sequelae for most of this century, the mechanism of radiation-induced salivary hypofunction remains unclear, and no corrective treatment is currently available. The surviving salivary epithelial cells are predominantly ductal, considered to be salt absorptive, relatively water impermeable, and incapable of generating salivary fluid.

As a novel strategy to treat this condition, we have chosen to apply gene transfer technology to alter postradiation surviving ductal cell function. In a normal salivary gland, acinar cells secrete an isotonic "primary" fluid, from which considerable NaCl is resorbed during passage through the ductal system. Although currently very little is known about ion transport pathways in salivary ductal segments (6, 7), at least four such components are considered to be present in ductal cell luminal membranes (6). These are an epithelial Na^+ channel (6, 8); a Cl^- channel, likely the cystic fibrosis transmembrane conductance regulator (6, 9); a Na^+/H^+ exchanger (6, 9); and a K^+/H^+ exchanger (6, 10). The first three components are believed to be important to NaCl resorption by ductal cells (6, 11). The K^+/H^+ exchanger, on the other hand, is thought to be responsible for the secretion of K^+ and HCO_3^-, both of which are typically found well above serum levels in the final saliva (6, 10).

In the absence of acinar cells, such as would occur after extensive therapeutic irradiation (2, 12), no primary fluid would be generated and, thus, the Na^+ channel, Cl^- channel and Na^+/H^+ exchanger would be practically inoperative. However, the luminal K^+/H^+ exchanger could continue to function and, in the presence of CO_2 (HCO_3^-, H^+), to secrete $KHCO_3$. Accordingly, we have speculated that this K^+/H^+ exchanger could contribute to the generation of an osmotic $KHCO_3$ gradient (luminal > interstitial), which would allow significant fluid movement into the lumen across ductal cells if a facilitated water permeability pathway, such as a water channel, was present. Currently, however, no known water channel has been identified in salivary intralobular and excretory duct membranes (13, 14).

Based on this rationale, we thought to introduce a facilitated water permeability pathway in salivary glands exposed to radiation via a recombinant adenovirus. Replication-deficient recombinant adenoviruses have been used for efficient gene transfer to mammalian epithelial cells *in vitro* and *in vivo* (15–19). Aquaporins (AQPs) are a recently described family of water channel proteins (13, 20, 21). The prototype is AQP1, a 28-kDa protein first isolated from human red blood cells (22–24). AQP1 is a constitutively activated water channel, which does not exhibit a polarized membrane distribution in most epithelia (13, 20, 21). Furthermore, within salivary glands, AQP1 is only detected in venules and capillaries, but not in parenchymal cells (14, 25).

In the present report, we describe the construction of a recombinant adenovirus encoding human AQP1 (hAQP1). We show that this virus can direct hAQP1 expression *in vitro* in several epithelial cell lines and *in vivo* in salivary gland parenchyma. When the virus is administered to rat salivary glands that have been irradiated and rendered either modestly or markedly hypofunctional, salivary flow rate is dramatically increased. Our findings suggest that hAQP1 gene transfer may provide a novel therapeutic approach for patients with postradiation salivary hypofunction.

METHODS

Construction of AdhAQP1. The hAQP1 cDNA (22) was directly cloned into the adenovirus shuttle vector pACCMV,

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Abbreviations: AQP, aquaporin; hAQP1, human aquaporin 1; MOI, multiplicity of infection; pfu, plaque-forming units.

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which was generously provided by C. Newgard (University of Texas Southwestern Medical Center, Dallas). To do this, PCR primers were designed to amplify the hAQP1 cDNA (≈3 kb) and to add restriction sites for cloning. The sense primer was 5′-GGCGGGTACCAGCTCTCAGAGGGAATT-GAGCACCCGGCAGCGGTCTCAGG-3′, and contained a *Kpn*I site and 19 nucleotides corresponding to the 5′ untranslated sequence that was not present in the plasmid cDNA. The antisense primer was 5′-GCCGGGATCCGGAAACAGC-TATGACCATG-3′, and contained a *Bam*HI site. PCR amplification was performed using the hAQP1 cDNA (22) as a template (1 min at 94°C, 1 min at 59°C, 2 min at 72°C, 35 cycles) and a GeneAmp PCR reagent kit (Perkin–Elmer). The PCR-amplified hAQP1 cDNA was inserted at the *Kpn*I and *Bam*HI sites of pACCMV to yield the plasmid pCMVhAQP1. pCMVhAQP1 was then cotransfected with pJM17, which contains the adenovirus type 5 sequence, in 293 cells as described (26) to yield AdhAQP1. AdhAQP1 is a replication-deficient recombinant adenovirus encoding hAQP1 and including a cytomegalovirus promoter and simian virus 40 polyadenylylation site. This virus was subsequently plaque-purified, grown in large quantity, and purified by CsCl gradient centrifugation (26). The titer of the virus, determined by limiting dilution plaque assay, was 4×10^{11} plaque-forming units (pfu)/ml.

Northern and Western Blot Analyses. Oligonucleotide primers were designed to amplify ≈813 bp of the hAQP1 cDNA as a probe for Northern blot analyses. The primers used were as follows: sense, 5′-ATGGCCAGCGAAATCAAGAA-3′; antisense, 5′-CCTCTATTTGGGCTTCATCTC-3′. PCR amplification was performed as above using 20 pmol of primers with Bluescript II (SK−) containing the hAQP1 cDNA (22) as template. A band of ≈813 bp was obtained after agarose gel electrophoresis. The PCR product was purified on a Select-D, G-50 column (5′ → 3′). Northern blots were performed using poly(A)$^+$ RNA essential as previously described (27). Western blot analyses, using an affinity-purified antibody against hAQP1 (dilution 1:1000; ref. 14), were performed on samples of cultured cell, or submandibular gland, membranes as previously reported (27, 28).

Localization and Function of hAQP1 in MDCK Cells. The distribution of hAQP1 in MDCK cells (strain MDCK-c, a cyst-forming clone, a gift from J. Grantham, University of Kansas) was determined by confocal microscopy as previously described (27), using the above affinity-purified antibody. Cells were grown to confluence on 24.5-mm Transwell-Col culture chambers (Costar). The secondary antibody used was a fluorescein isothiocyante-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Samples were examined in a Nikon Optiphot photomicroscope equipped with a Bio-Rad MRC-1000 laser scanning confocal imaging system (Bio-Rad), using a Krypton/Argon laser as light source. The net fluid secretion across polarized MDCK cell monolayers was determined using a modification of the technique of Neufeld *et al.* (29) as reported earlier (27). The apical fluid medium was replaced by a hyperosmotic medium (400 mosm), and fresh isosmotic medium was placed at the basal side. After 4 h, the liquid in the apical chamber was collected with a calibrated pipette, and net fluid movement (water flow) determined.

Immunohistochemical Detection and Localization of hAQP1 in Rat Submandibular Glands. hAQP1 localization in submandibular glands was determined by immunohistochemistry using the affinity-purified antibody to hAQP1 (dilution 1:100) and a Histostain SP kit (Zymed). Sections from paraffin-embedded submandibular glands were prepared from both irradiated (see below) and sham-irradiated glands, which had been infected with 5×10^9 pfu of either Addl312 (a control, replication-deficient adenovirus that encodes no transgene, ref. 26) or AdhAQP1. Quantitation of the proportion of cells expressing hAQP1 in control and AdhAQP1-infected glands was achieved by counting immunopositive and total cells in 4–6 high-power fields using gland pairs from two or three animals and an optical grid. All cell counting was done by one examiner.

Rat Irradiation, Gene Transfer, and Saliva Collection and Analysis. Male Wistar rats (250–300 g) were irradiated with a single dose of either 17.5 or 21 Gy, with the ventral surface of their necks exposed to the source. Control animals were sham-irradiated, i.e. anesthetized and placed in the irradiator, but not exposed to ionizing radiation. After 3 months (17.5 Gy) or 4 months (21 Gy), all rats were subjected to *in vivo* adenoviral-mediated gene transfer (5×10^9 pfu of either Addl312 or AdhAQP1) to the submandibular glands by retrograde ductal instillation (19, 30). Animals received 1 mg of dexamethasone (i.m. injection) at the time of gene transfer and each day until saliva collection to suppress inflammation, due to adenoviral infection, and preserve salivary function (30). Saliva was collected 3 days after this infection. The submandibular glands were again cannulated and saliva collected for 10 min after retrograde injection of 25 μl of pilocarpine (5 mg/ml), and for an additional 5 min after intramuscular injection of pilocarpine (5 mg/kg). Saliva volumes were determined gravimetrically after the 15-min collection. Na^+ and K^+ concentrations in rat submandibular saliva samples were determined as previously described (31).

RESULTS

AdhAQP1-Mediated hAQP1 Expression *in Vitro*. AdhAQP1 was constructed by homologous recombination between pCMVhAQP1 and pJM17 plasmids, and its structure verified by restriction and sequence analyses. The ability of AdhAQP1 to direct the expression of hAQP1 was initially assessed in 293 cells (human kidney) *in vitro* by both Northern blot and Western blot analyses. Northern blot analysis showed no hAQP1 transcript present in uninfected 293 cells (data not shown) or in 293 cells infected for 24 h with an adenovirus expressing α-1 antitrypsin (Adα1AT; see ref. 15; Fig. 1*A*). However, in AdhAQP1-infected 293 cells, abundant amounts of a hybridization-positive transcript of ≈3 kb were detected 24 h postinfection (Fig. 1*A*). This is the length of the transcript expected for hAQP1 (22). Western blot analysis of AdhAQP1-infected 293 cells showed the presence of both the nonglycosylated (≈28 kDa) and glycosylated (≈35–45 kDa) forms of

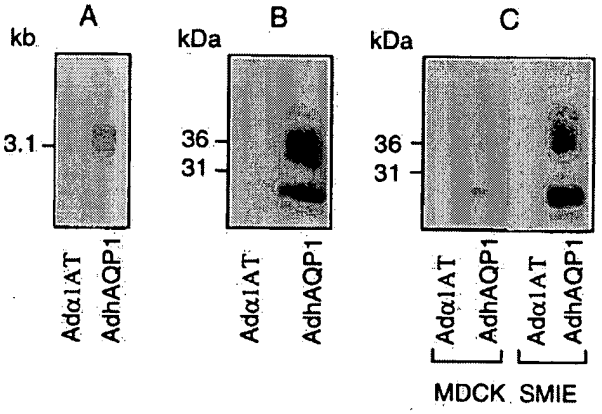

FIG. 1. hAQP1 expression in 293 cells and other epithelial cells. (*A*) Northern blot analysis was performed using 10 μg of total RNA, and 293 cells were infected at a MOI of 100 with either Adα1AT or AdhAQP1 and hybridized with a 813-bp radiolabeled probe of hAQP1 as described in *Methods*. (*B*) Ten micrograms of crude membranes from 293 cells infected at a MOI of 100 with either Adα1AT or AdhAQP1 were analyzed by Western blot as described in *Methods*. (*C*) Ten micrograms of crude membranes from MDCK and SMIE cells were analyzed by Western blot as above. The cells were infected at a MOI of 100 with either Adα1AT or AdhAQP1.

hAQP1 (Fig. 1*B*) (22, 23). Additionally, we have observed that AdhAQP1 was able to drive hAQP1 expression in other epithelial cell lines, e.g. MDCK (derived from dog kidney) and SMIE (derived from rat submandibular) cells (Fig. 1*C*).

Sorting and Function of hAQP1 in MDCK Cells After AdhAQP1 Infection. MDCK cells were used to study the sorting and function of the hAQP1 protein after AdhAQP1 infection. These cells are able to form polarized monolayers with distinct apical and basolateral membranes when grown on collagen-coated permeable filters (32). Uninfected MDCK cells (not shown) and cells infected with Adα1AT (Fig. 2*A* and *B*) at a multiplicity of infection (MOI) of 100 showed no hAQP1 expression. Conversely, cells infected with AdhAQP1 exhibited hAQP1 immunolabeling at both the apical and basolateral membranes (Fig. 2 *C* and *D*), similar to its distribution *in vivo* for several epithelial and endothelial cells (13, 20, 21).

We used MDCK cells as an *in vitro* model to determine if the AdhAQP1-directed hAQP1 protein expression led to increased water permeability (27, 29). The net movement of fluid across MDCK cells was measured in the presence of a transepithelial (apical > basal) osmotic gradient. After 4 h incubation, the net fluid movement across uninfected cells, or cells infected with Adα1AT, was similar, approximately 1 $\mu l/cm^2$ per h. However, in AdhAQP1-infected MDCK cells net fluid movement was increased ≈4-fold (Fig. 3), demonstrating that the expressed hAQP1 was functional.

AdhAQP1-Mediated hAQP1 Expression *in Vivo* in Rat Submandibular Glands. To determine if AdhAQP1 infection could lead to hAQP1 expression *in vivo*, we administered AdhAQP1 to rat submandibular glands via retrograde ductal instillation. After 24 h we prepared membranes from these glands and from glands either infected with Addl312, or uninfected. Membranes from uninfected rat submandibular glands (data not shown) or glands infected with Addl312 showed evidence of some nonglycosylated and glycosylated forms of AQP1 (Fig. 4*A*). This represents the endogenous rat AQP1, which is known to be present in blood vessels within the gland (Fig. 5*A*; see ref. 14). After infection with AdhAQP1, AQP1 levels in membranes from whole glands were increased ≈5-fold as judged from Western blot analyses (Fig. 4*A*). We also obtained samples of control and AdhAQP1-infected glands for localization of hAQP1 in various gland cell types. In nonirradiated glands infected with Addl312, 2.1 ± 0.5% (mean ± SEM) of the cells were immunopositive for AQP1 (these represent endothelial cells in capillaries and venules; refs. 14, 25). Similarly, Addl312-infected, irradiated (17.5 Gy) glands showed low levels of cellular immunolabeling (1.6 ± 0.3%). AdhAQP1-infected glands showed high AQP1 immunolabeling levels in both acinar and ductal cells (Fig. 5*B*). In AdhAQP1-infected nonirradiated glands 15.0 ± 3.6% of the cells were immunopositive; a significant increase above that

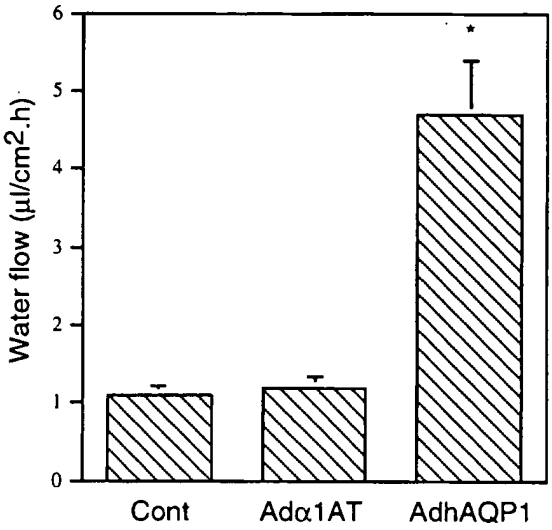

FIG. 3. Net fluid secretion rate of MDCK cells. Net fluid secretion rate of control, Adα1AT-(MOI of 100)- and AdhAQP1-(MOI of 100)-infected MDCK cells was measured as described in *Methods*. The results are expressed as water flow in microliters of fluid secreted per cm^2 per h and are the mean ± SEM of three separate experiments, each performed in triplicate. The results were analyzed using an ANOVA. *, $P < 0.05$ compared with control and Adα1AT-infected cells.

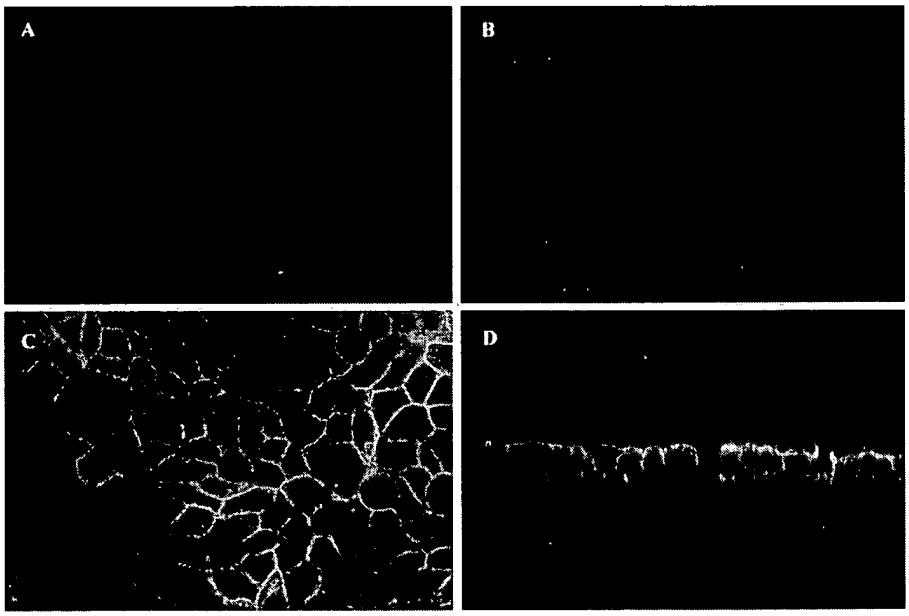

FIG. 2. Localization of hAQP1 in polarized MDCK cells. Confluent MDCK cells, grown on filters, were infected for 24 h at the basolateral side at a MOI of 100 with either Adα1AT (*A* and *B*) or AdhAQP1 (*C* and *D*). hAQP1 expression was determined as described in *Methods*. Micrographs of horizontal (*xy*; *A* and *C*), and vertical (*xz*; *B* and *D*) optical sections through MDCK cells are shown.

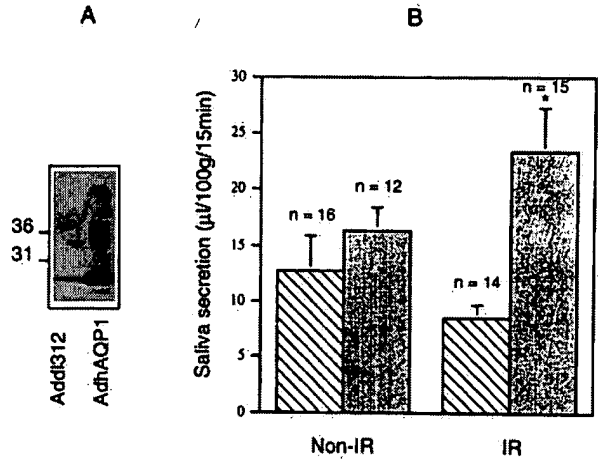

FIG. 4. Expression and function of the recombinant hAQP1 *in vivo*. (*A*) Ten micrograms of crude membranes from rat submandibular glands, which were cannulated and infected for 24 h with 5×10^9 pfu of either Addl312 or AdhAQP1, were used for Western blot analysis as in Fig. 1. (*B*) Submandibular glands of sham-irradiated (Non-IR) or irradiated (IR) rats were cannulated and retrograde instilled with 5×10^9 pfu of either Addl312 (hashed bar) or AdhAQP1 (solid bar), and then saliva was collected after 3 days as described in *Methods*. The results are expressed as saliva secretion in microliters per 100 g of rat body weight per 15 min and are the mean ± SEM. The results were analyzed using an ANOVA. *, $P < 0.05$ between the two groups with different instilled viruses.

seen in Addl312-infected glands ($P < 0.05$). In irradiated glands infected with AdhAQP1, the proportion of cells expressing AQP1 was significantly greater (33.6 ± 1.34%; $P < 0.05$)

Saliva Secretion from AdhAQP1-Infected Rat Submandibular Glands. We next determined if the expression of hAQP1 occurring subsequent to AdhAQP1 infection was associated with increased salivary fluid secretion. We first examined salivary secretion from rat submandibular glands that were either sham-irradiated or given a single exposure of 17.5 Gy. Three months postsham or actual irradiation, all animals were infected with either Addl312 or AdhAQP1. As shown in Fig. 4*B*, when compared with sham-irradiated rats, rats exposed to radiation and administered Addl312 exhibited a modest, ≈30%, but not statistically significant, reduction in saliva production. We next compared the effect of AdhAQP1 infection on salivary secretion in sham-irradiated and irradiated animals. AdhAQP1 had a modest, but not significant, effect on salivary secretion in the sham-irradiated animals. However, in the irradiated rats, AdhAQP1 infection resulted in a higher level of saliva production. Salivary secretion in these animals was ≈3-fold ($P < 0.05$) that observed from glands of irradiated animals infected with the control virus (Addl312).

Saliva also was collected from submandibular glands of an additional set of rats that were either sham-irradiated or given a single radiation dose of 21 Gy (Table 1), a condition expected to result in greater salivary hypofunction. In this group of animals, infection with either Addl312 or AdhAQP1 was performed at 4 months postirradiation. Saliva secretion from the sham-irradiated and Addl312-infected glands was 36.6 ± 6.8 μl/100 g. Salivary secretion from irradiated glands infected with Addl312 was significantly lower, by 64% ($P < 0.05$; 13.2 ± 3.7 μl/100 g) than from the sham-irradiated group, consistent with greater salivary hypofunction. AdhAQP1 infection of sham-irradiated glands was without effect on salivary secretion (28.4 ± 8.0 μl/100 g) compared with that seen with Addl312-infected glands (above). However, AdhAQP1-infection of irradiated glands resulted in a ≈2-fold increase in submandibular gland secretion (30.6 ± 3.5 μl/100 g) compared with Addl312-infected glands (above; $P < 0.05$).

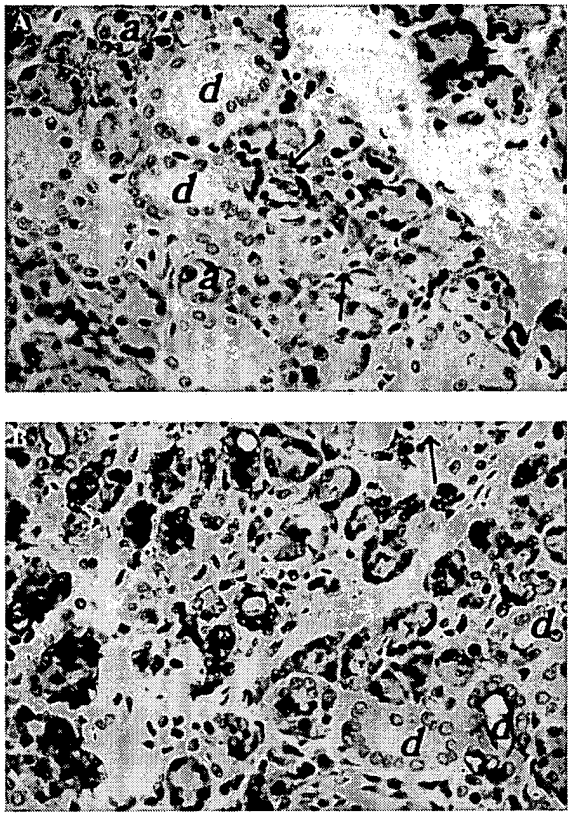

FIG. 5. Localization of hAQP1 in rat submandibular glands infected with AdhAQP1. hAQP1 localization was determined by immunohistochemistry (see *Methods*) on paraffin sections from sham-irradiated rat submandibular glands infected with 5×10^9 pfu of either Addl312 (*A*) or AdhAQP1 (*B*). Arrows indicate endothelial cells in capillaries and venules. a, acinar cells; d, ductal cells. Similar results were found with irradiated glands (not shown).

DISCUSSION

We have constructed a recombinant adenovirus, AdhAQP1, and shown that it mediated hAQP1 expression in membranes of epithelial cell lines from several species (human, dog, rat) *in vitro*. The recombinant hAQP1 protein exhibited several characteristics typical of native hAQP1 (13, 20, 21): (*i*) the presence of nonglycosylated and glycosylated forms; (*ii*) a nonpolarized membrane distribution; and (*iii*) the ability to mediate the transepithelial movement of fluid. AdhAQP1 also

Table 1. Effect of AdhAQP1 infection on secretion by rat submandibular glands irradiated with 21 Gy

	Saliva secretion after viral infection, μl/100 g body weight per 15 min	
Experimental group	Addl312	AdhAQP1
Sham-irradiation	36.6 ± 6.8[a] ($n = 4$)	28.4 ± 8.0[c] ($n = 6$)
21 Gy irradiation	13.2 ± 3.7[a,b,c] ($n = 6$)	30.6 ± 3.5[b] ($n = 9$)

Animals were either sham-irradiated or their salivary glands were exposed to a single radiation dose of 21 Gy. Four months later, either a control virus (Addl312) or AdhAQP1 was administered via retrograde ductal instillation to the submandibular glands. Three days later saliva was collected from these glands. The numbers in parentheses represent the number of animals in each treatment group. Values are mean ± SEM; values with the same superscript letter are significantly different, $P < 0.05$, by a Student's *t* test.

mediated hAQP1 expression in rat submandibular gland membranes *in vivo*. Both acinar and ductal cell types were targets for the virus. This wide distribution is consistent with our earlier studies using an adenovirus encoding a reporter gene (β-galactosidase) to evaluate infected cell populations (19).

The impetus for this study was to test a novel strategy for increasing fluid secretion in human salivary glands exposed to ionizing radiation. As noted above, in humans, such radiation especially damages the primary fluid secreting acinar cells, whereas the ductal cells are much less affected. For patients this can result in a marked reduction of salivary flow (4, 5). We used rat salivary glands, which are a convenient and often-used model to study secretory irradiation damage, to test this strategy. Although rat salivary glands are considered relatively more radioresistant than primate and human glands (2), at higher cumulative radiation doses and longer postradiation times rat salivary glands show similar morphological changes (loss of acinar cells) and marked secretory dysfunction (33–36). In the present study, three months after exposure to 17.5 Gy, rats exhibited a modest reduction (≈30%) in salivary flow, but no striking morphological changes in their glands. Nonetheless, salivary secretion was markedly increased if the irradiated glands were infected with AdhAQP1 versus the control virus Addl312. When salivary glands were exposed to a higher radiation dose (21 Gy), and salivary secretion measured 4 months postirradiation, salivary flow rates were dramatically lower (64%), and the submandibular gland parenchyma exhibited clear morphological changes (data not shown). These results are consistent with previous observations, reflecting an experimental situation more like that observed in irradiated patients (e.g., refs. 12, 33, 35). Most importantly, administration of AdhAQP1 to these more severely affected rat salivary glands was able to enhance salivary secretion significantly, approaching levels seen with sham-irradiated glands infected with control virus (30.6 ± 3.5 μl/100 g vs. 36.6 ± 6.8 μl/100 g, respectively). Thus, the strategy suggested above for increasing fluid secretion from irradiated human salivary glands was able to increase fluid secretion from irradiated rat salivary glands.

At present the exact mechanism by which this increase in fluid secretion from irradiated rat salivary glands occurs is unknown. The hypothesis described above, i.e. fluid movement in response to a $KHCO_3$-generated osmotic gradient, is speculative. We have, however, determined the $[K^+]$ and $[Na^+]$ in the saliva collected from irradiated submandibular glands infected with either Addl312 or AdhAQP1. Although this saliva reflects a mixture of fluid output from all parenchymal tissue present, some of which may be more severely affected by radiation than others, interestingly we found that $[K^+]$ in saliva from animals administered AdhAQP1 had >40% higher $[K^+]$, 99.1 ± 7.4 mM, than the $[K^+]$ found in saliva of animals infected with Addl312 (69.6 ± 10.6; $P < 0.05$). Conversely, no difference was observed in salivary $[Na^+]$ between these two groups (14.1 ± 2.9 vs. 15.1 ± 2.7 mM). While these results are generally in keeping with the above hypothesis, considerably more study is necessary to understand the mechanism for AdhAQP1-mediated increases in fluid secretion from irradiated glands. An additional, but unexpected, finding was that we observed that more (≈2-fold) glandular parenchymal cells were transduced by AdhAQP1 in irradiated glands than in sham-irradiated glands. The reason for this difference is not known, but it was a consistent finding in all animals examined and likely contributed to the increased saliva secretion measured.

There are two current, significant, and presently inescapable limitations to fully understanding the results presented here. First, relatively little is known about the physiology of salivary ductal cells, and what is known represents a mixture of findings from different ductal segments (intralobular; main excretory; refs. 6–10). Second, the exact nature of radiation damage to salivary glands (human and rodent) is not clear and, indeed, is considered enigmatic (3, 36–40). Obviously, designing a specific molecular approach to alter ductal cell function requires detailed knowledge of the molecules functioning normally and pathologically, a situation presently impossible to realize. What is clear, however, is that AQP1 gene transfer to both modestly affected and significantly dysfunctional, irradiated rat submandibular glands leads to enhanced salivary secretion from these glands. These findings, obtained with two separately irradiated and treated cohorts of animals, strongly support the possibility of using gene therapy to correct the defects in salivary glands that occur subsequent to therapeutic irradiation. This approach may have the potential to improve salivary function and relieve the considerable morbidity experienced by patients.

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1. Silverman, S., Jr. (1992) *Clin. Geriatric Med.* **8,** 529–541.
2. Stephens, L. C., King, G. K., Peters, L. J., Ang, K. K., Schultheiss, T. E. & Jardine, J. H. (1986) *Am. J. Pathol.* **124,** 469–478.
3. Vissink, A. Kalicharan, D., s'Gravenmade, E. J., Jongbloed, W. L., Ligeon, E. E., Nieuwenhuis, P. & Konings, W. T. (1991) *J. Oral Pathol. Med.* **20,** 449–456.
4. Franzen, L., Funegard, U., Ericson, T. & Henriksson, R. (1992) *Eur. J. Cancer* **28,** 457–462.
5. Johnson, J. T., Ferretti, G. A., Nethery, W. J., Valdez, I. H., Fox, P. C., Ng, D., Muscoplat, C. C. & Gallagher, S. C. (1993) *N. Engl. J. Med.* **329,** 390–395.
6. Cook, D. I., Van Lennep, E. W., Roberts, M. L. & Young, J. A. (1994) in *Physiology of the Gastrointestinal Tract*, ed. Johnson, L. R. (Raven, New York), pp. 1061–1117.
7. Zhao, H., Xu, X., Diaz, J. & Muallem, S. (1995) *J. Biol. Chem.* **270,** 19599–19605.
8. Moran, A., Davis., V. H. & Turner, R. J. (1995) *Am. J. Physiol.* **268,** C350–C355.
9. He, X., Tse, C.-M., Donowitz, M., Alper, S. L., Gabriel, S. E. & Baum, B. J. (1997) *Pflügers Arch.* **433,** 260–268.
10. Knauf, H., Lubcke, R., Kreutz, W. & Sachs, G. (1982) *Am. J. Physiol.* **242,** F132–F139.
11. Turner, R. J. (1993) *Ann. N.Y. Acad. Sci.* **694,** 24–35.
12. Kashima, H. K., Kirkham, W. R. & Andrews, J. R. (1965) *Am. J. Roentgenol.* **94,** 271–291.
13. Agre, P., Brown, D. & Nielsen, S. (1995) *Curr. Opin. Cell Biol.* **7,** 472–483.
14. Li, J., Nielsen, S., Dai, Y., Lazowski, K. W., Christensen, E. I., Tabak, L. A. & Baum, B. J. (1992) *Pflügers Arch.* **428,** 455–460.
15. Rosenfeld, M. A., Siegfried, W. Yoshimura, K., Yoneyama, K., Fukuyama, M., Stier, L. E., Paako, P. K., Gilardi, P., Stratford-Perricaudet, L. P., Perricaudet, M., Jallat, S., Pavirani, A., Lecocq, J. P. & Crystal, R. G. (1991) *Science* **252,** 431–434.
16. Gomez-Foix, A. M., Coats, W. S., Baque, S., Alam, T., Gerard, R. D. & Newgard, C. B. (1992) *J. Biol. Chem.* **267,** 25129–25134.
17. Mitani, K., Graham, F. L. & Caskey, T. (1994) *Hum. Gene Ther.* **5,** 941–948.
18. Kozarsky, K. F., McKinley, D. R., Austin, L. L., Raper, S. E., Stratford-Perricaudet, L. D. & Wilson, J. (1994) *J. Biol. Chem.* **269,** 13695–13702.
19. Mastrangeli, A., O'Connell, B., Aladib, W., Fox, P. C., Baum, B. J. & Crystal, R. G. (1994) *Am. J. Physiol.* **266,** G1146–G1155.
20. Verkman, A. S., Van Hoek, A. N., Ma, T. Frigeri, A., Skach, W. R., Mitra, A., Tamarapoo, B. K. & Farinas, J. (1996) *Am. J. Physiol.* **270,** C12–C30.
21. Knepper, M. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6255–6258.
22. Preston, G. M. & Agre, P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11110–11114.
23. Denker, B. M., Smith, B. J., Kuhajda F. P. & Agre, P. (1988) *J. Biol. Chem.* **263,** 15634–15642.
24. Preston, G. M., Carroll, T. P., Guggino, W. B. and Agre, P. (1992) *Science* **256,** 385–387.

25. Nielsen, S., Smith, B. L., Christensen, E. I. & Agre, P. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7275–7279.
26. Becker, T. C., Noel, R. J., Coats, W. S., Gomez-Foix, A. M., Alam, T., Gerard, R. D. & Newgard, C. B. (1994) *Methods Cell. Biol.* **43,** 161–189.
27. Delporte, C., O'Connell, B. C., He, X., Ambudkar, I. S., Agre, P. & Baum, B. J. (1996) *J. Biol. Chem.* **271,** 22070–22075.
28. Lazowski, K. W., Li, J., Delporte, C. & Baum, B. J. (1995) *J. Cell. Physiol.* **164,** 613–619.
29. Neufeld, T. K., Grant, M. E. & Grantham, J. J. (1991) *J. Tiss. Cult. Meth.* **13,** 229–234.
30. Adesanya, M., Redman, R. S., Baum, B. J. & O'Connell, B. C. (1996) *Hum. Gene Ther.* **7,** 1085–1093.
31. Bodner, L., Qwarnstrom, E., Omnell, K.-A., Hand, A. R. & Baum, B. J. (1983) *Comp. Biochem. Physiol.* **74A,** 829–831.
32. Cereijido, M., Robbins, E. S., Dolan, W. J., Rotunno, C. A. & Sabatini, D. D. (1978) *J. Cell Biol.* **77,** 853–880.
33. Cherry, C. P. & Glucksmann, A. (1959) *Brit. J. Radiol.* **32,** 596–608.
34. Chomette, G., Auriol, M., Vaillant, J. M., Bertrand, J. Ch. & Chenal, Ch. (1981) *Virchows Arch. Pathol. Anat.* **391,** 291–299.
35. Franzen, L., Gustaffson, H., Sundstrom, S., Karlson, M., Littbrand, B. & Henriksson, R. (1993) *Int. J. Radiat. Biol.* **64,** 93–101.
36. Nagler, R. M., Baum, B. J. & Fox, P. C. (1993) *Radiat. Res.* **136,** 392–396.
37. Stephens, L. C., Schultheiss, T. E., Ang, K. K. & Peters, L. J. (1989) *Cancer Bull.* **41,** 106–114.
38. Mertz, P. M., Fox, P. C., Pluta, A., Baum, B. J. & Kouslevari, E. E. (1992) *Radiat. Res.* **130,** 104–112.
39. Peter, B., Van Waarde, M. A. W. H., Vissink, A., 's-Gravenmade, E. & Konings, A. W. T. (1994) *Radiat. Res.* **140,** 257–265.
40. Nagler, R., Marmary, Y., Fox, P. C., Baum, B. J., Har-El, R. & Chevion, M. (1997) *Radiat. Res.* in press.

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Adenoassociated Virus-Mediated Transfer of a Functional Water Channel into Salivary Epithelial Cells *In Vitro* and *In Vivo*

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ABSTRACT

Aquaporin 1 (AQP1) is the archetypal member of a family of integral membrane proteins that function as water channels. Previously we have shown that this protein can be expressed transiently from a recombinant adenovirus (AdhAQP1) *in vitro* in different epithelial cell lines, and *in vivo* in rat submandibular glands. In the present study we have constructed a recombinant adenoassociated virus (rAAV) containing the human aquaporin 1 gene (rAAVhAQP1). rAAVhAQP1 was produced at relatively high titers. $\approx 10^{11}$–10^{12} particles/ml and $\approx 10^{8}$–10^{9} transducing units/ml. We show that the rAAVhAQP1 can transduce *in vitro* four epithelial cell lines of different origins, at a level sufficient to detect the recombinant hAQP1 protein by either Western blot or confocal microscopic analysis. The recombinant hAQP1 was correctly targeted to the plasma membranes in all cell lines. Function of the recombinant hAQP1 was measured as fluid flow, in response to an osmotic gradient, across a monolayer of transduced epithelial cells. The data show that even at a low level of transduction, typically ≈10% of the cells in the monolayer, transepithelial fluid movement is enhanced about threefold above basal levels. In addition, we report that rAAVhAQP1 can transduce epithelial cells in the salivary glands and liver of mice *in vivo*. These results suggest that rAAVs may be useful gene transfer vectors to direct the production of functional transgenes in salivary epithelial cell types.

OVERVIEW SUMMARY

Previously, we have shown several potential clinical uses for gene transfer to salivary glands, using adenoviral vectors. However, adenovirus-mediated gene transfer leads to transient transgene expression and a significant host immune response. As an alternative means to deliver genes to salivary epithelial cells, we have begun to explore the use of recombinant adenoassociated viruses (rAAVs). In this article we describe the construction of an rAAV that directs the expression of functional aquaporin 1, *in vitro* and *in vivo*.

INTRODUCTION

OUR LABORATORY has demonstrated that it may be possible to utilize gene transfer technology to treat the severe damage to salivary gland secretory cells that results from therapeutic radiation for head and neck cancer (Delporte *et al.*, 1997a). To demonstrate this principle, we used a replication-deficient adenoviral vector (AdhAQP1) encoding human aquaporin 1 (hAQP1), the archetypal mammalian water channel (Preston and Agre, 1991). Adenovirus-mediated expression of hAQP1 in irradiated glands led to a two- to threefold increase in salivary fluid secretion compared with glands administered a control virus (Delporte *et al.*, 1997a). While recombinant adenoviruses offer the possibility of high *in vivo* transduction efficiencies (Kozarsky and Wilson, 1993; Mulligan, 1993), the presently available adenoviral vectors do not lead to stable, long-term expression of the transgene and result in a potent immunologic response. The latter both destroys transgene expressing cells through the action of cytotoxic T lymphocytes and prevents successful vector readministration because of the production of neutralizing antibodies (e.g., Adesanya *et al.*, 1996; Jooss *et al.*, 1996; Smith *et al.*, 1996; Yang *et al.*, 1996; Mack *et al.*, 1997; Worgall *et al.*, 1997; Kagami *et al.*, 1998).

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Adenoassociated virus (AAV) is currently being investigated as a possible alternative to adenoviral gene therapy vectors, and other methods of gene transfer (Carter, 1992; Kotin, 1994). A member of the Parvoviridae family, AAV is the smallest DNA virus known to infect humans. There are many features of AAV that make it an attractive candidate for gene therapy. These features include a broad host range, no association with any disease, and the ability of wild-type AAV to integrate into the host genome.

Wild-type (wt) AAV is capable of infecting both quiescent cells and actively dividing cells (Russell *et al.*, 1994; McKeon and Samulski, 1996). Recombinant AAV (rAAV) vectors appear to lack site-specific integration, which is a feature of wt AAV (Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997). The single-stranded, 4.6-kb AAV genome has inverted terminal repeats (ITRs) that contain the *cis* sequences required for replication and packaging (McLaughlin *et al.*, 1988), and mediate the integration of AAV into the host genome (Samulski *et al.*, 1989). The presence of the virally coded Rep protein directs AAV integration to a specific locus on human chromosome 19 (Kotin *et al.*, 1990). This biological property may eventually allow for the stable, long-term expression of a foreign gene in many different cell types. In fact, sustained expression from an rAAV has been shown *in vivo* in several tissues (e.g., Alexander *et al.*, 1996; Conrad *et al.*, 1996; Kessler *et al.*, 1996; Xiao *et al.*, Flannery *et al.*, 1997; Snyder *et al.*, 1997).

Because of the unique features offered by AAV-mediated gene transfer, we have begun to explore the possibility of using rAAV as a vector to mediate gene transfer to salivary gland epithelial cells. In the present study, we report the construction of an rAAV expressing hAQP1 (rAAVhAQP1). We have examined the ability of this rAAV to transduce various epithelial cell lines *in vitro* as well as epithelial cells in the salivary glands and liver of mice *in vivo*.

MATERIALS AND METHODS

Cells and cultures conditions

Four mammalian epithelial cell lines were employed in the present study. Human embryonic 293 cells and monkey COS cells are derived from kidney. HSG (Shiarasuna *et al.*, 1981) and SMIE (He *et al.*, 1990) cells are derived from human and rat submandibular glands, respectively. All cells were maintained in monolayer cultures in medium with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 m*M* glutamine.

Plasmids

pAAVhAQP1 was derived from pCMVhAQP1 (Delporte *et al.*, 1997a). pAAV-MCS2.7 was derived from pAV2 by inserting a synthetic oligonucleotide into the *PmlI* site (nucleotide 209 of AAV) and in the *SnaBI* site (nucleotide 4492). A 3.6-kb fragment containing the cytomegalovirus (CMV) promoter, hAQP1 open reading frame, and simian virus 40 (SV40) poly(A) signal was released from pCMVhAQP1 by digestion with *NotI* and the ends filled in using Klenow enzyme. The vector backbone and AAV ITRs were obtained from pAAV-MCS2.7 by digestion with *PmlI* and *Sna*BI. The blunt-ended fragments were ligated and orientation of the insert determined using restriction enzyme fragment mapping.

rAAV production and titer

rAAVhAQP1 was produced using pAAVhAQP1 in a high-titer system as described previously (Chiorini *et al.*, 1995). Fractions collected from CsCl gradients were assayed for rAAV genomes by dot-blot hybridization (Flotte *et al.*, 1992). Genome titer was determined by comparison of signal intensity with that of known amounts of pCMVhAQP1 as a standard. We assessed the titer of wt AAV in our rAAV preparations by using the polymerase chain reaction. Reaction mixes contained ~100 ng of DNA and employed 25 pmol of Rep-specific primers. The upstream primer was ATGCCGGGGTTTTACGAGATTGTG and the downstream primer was CGCCCACTGGAGCTCAGGCTGGGTT. Reaction mixes were denatured for 2 min at 95°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 55°C, and 1 min at 72°C. This procedure yields a 490-bp product.

Membrane preparation

Crude plasma membranes were obtained essentially as described previously (Delporte *et al.*, 1996). Briefly, transduced and control cells were collected in 1 m*M* $NaHCO_3$ and subjected to a freeze–thaw cycle, and cell debris was collected by centrifugation at 6000 × *g*. The resulting supernatant was then centrifuged at 16,000 × *g* and the pellet, resuspended in 50 m*M* Tris-HCl (pH 7.5), was used as the membrane source.

Western blot analysis

Membrane proteins were resolved on a 12% Tris–glycine–sodium dodecyl sulfate (SDS) gel and transferred to a polyvinyl difluoride membrane, using the X-Cell system (Novex, San Diego, CA). Western blots were performed as described (Delporte *et al.*, 1996, 1997a), using an affinity-purified antibody to hAQP1 (Li *et al.*, 1994) at a 1:1000 dilution. The secondary antibody was a goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, (CA) used at a 1:5000 dilution. Bound antibodies were detected by the enhanced chemiluminescence (ECL) method (Amersham, Arlington Heights, IL).

Functional assay

SMIE cells were grown to confluence on collagen-coated polycarbonate filter inserts in six-well plates (Costar, Cambridge, MA). After the cells formed a confluent monolayer, they were transduced with either rAAVhAQP1 or rAAVAP (the latter encoding the alkaline phosphatase gene), at a multiplicity of infection (MOI) of 100. For rAAV studies herein, MOI refers to the number of particles per cell. rAAV transductions were performed with and without coinfection of wild-type adenovirus at an MOI of 100. Transductions with the recombinant adenovirus encoding AQP1, AdhAQP1, were performed at an MOI of 100 (Delporte *et al.*, 1997a). For adenovirus studies herein, MOI refers to the number of plaque-forming units per cell. Net fluid secretion across the SMIE cell monolayer was measured as described (Delporte *et al.*, 1998; He *et al.*, 1998), using a hyperosmotic medium (440 mOsm) in the apical chamber and isoosmotic culture medium (340 mOsm) in the basal chamber.

Confocal microscopy

Cells were grown on filter inserts or glass coverslips, as described above, fixed with ice-cold 100% methanol, and subjected to immunofluorescent staining for hAQP1 protein as described (Delporte *et al.*, 1996, 1997a), using the affinity-purified antibody described above. Samples were examined using a Nikon Optiphot-2 photomicroscope equipped with a Bio-Rad MRC-1000 laser scanning confocal imaging system (Bio-Rad, Cambridge, MA), with a krypton–argon laser as light source.

In vivo *experiments*

Male BALB/c mice were anesthetized by intramuscular injection of a mixture of ketamine chloride (60 mg/kg) and xylazine (5 mg/kg). Atropine (0.5 mg/kg) was administered intramuscularly to decrease salivary flow. Delivery of virus, at 10^8 particles per 50 μl of buffer (10 m*M* Tris [pH 7.4], 0.1 m*M* $MgCl_2$, 10% glycerol), to the submandibular glands was done by retrograde ductal instillation as previously reported for adenoviral vectors in rats (Delporte *et al.*, 1997a,b). For comparison, an equivalent dose of rAAVhAQP1 was also administered by tail vein injection. Tissues were harvested after 4 weeks, embedded in O.C.T. compound (VWR, Bridgeport, NJ), and stored at −80°C. Similar studies were attempted in male Wistar rats; however, we were unable to demonstrate convincing transduction of salivary epithelial cells by rAAVhAQP1 using these methods.

Immunohistochemistry

Frozen samples were fixed for 5 min in ice-cold 100% acetone, rehydrated with a graded series of ethanol washes, and rinsed in phosphate-buffered saline (PBS) before detection using the primary antibody already described and the Histostain SP kit (Zymed, San Francisco, CA).

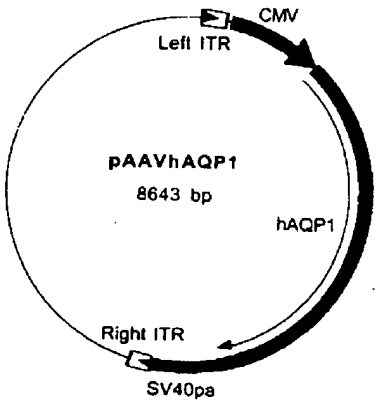

FIG. 1. Plasmid map. pAAVhAQP1 contains the AAV left and right ITRs flanking the CMV promoter, hAQP1 coding sequences, and SV40 polyadenylation signal.

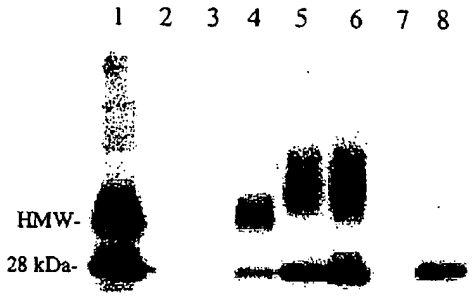

FIG. 2. Detection of hAQP1 in epithelial cell membranes by Western blot. Lane 1, 1 μg of a crude rat kidney membrane preparation (positive control). 293, COS, and HSG cells were either untreated (lanes 3, 5, and 7, respectively) or transduced with rAAVhAQP1 at an MOI of 100 (lanes 4, 6, and 8, respectively) and the expression of hAQP1 protein examined in crude membrane preparations using an affinity-purified antibody to hAQP1. Lane 2 is unlabeled molecular weight standards. Endogenous expression of AQP1 (monkey homolog) was detected only in COS cells (lane 5). 28 kDA, Nonglycosylated monomeric AQP1; HMW, high molecular weight glycosylated AQP1.

RESULTS

Analysis of rAAVhAQP1 preparations

Figure 1 provides a schematic diagram of the construction of pAAVhAQP1. For the experiments shown in this article, five separate preparations of the recombinant virus were used. An analysis of the virus particle titer, i.e., number of genomes, showed an average of 5×10^{11} genomes/ml. In our preparative scheme the rAAV was typically found at highest concentrations in fractions with a density of 1.4 g/ml. The peak rAAV genome-containing fractions were heated at 55°C for 1 hr to inactivate any wild-type adenovirus present. No adenoviral infectivity was detected in any of these heat-inactivated fractions, as measured by the induction of cytopathological morphology in 293 cells, even after 2 weeks of incubation. Transduction assays with rAAV indicated an average ratio of 1:1000 (transducing particles:total particles). Using a polymerase chain reaction method to determine the titer of wt AAV in our rAAV preparations, we could detect ~1 wt AAV in 10^5 rAAV particles.

Detection of hAQP1 in membranes from transduced epithelial cells

Neither 293 cells nor HSG cells express hAQP1 endogenously (Fig. 2, lanes 3, 7). COS cells (lane 5), however, showed immunoreactive AQP1 bands: a distinct band at ~28 kDa corresponding to the AQP1 monomer and a broader band (~35–45 kDa) corresponding to the glycosylated AQP1 (Denker *et al.*, 1988).

Following transduction with rAAVhAQP1, membranes from all studied epithelial cell lines showed substantial levels of hAQP1 protein by Western blot analysis. As a positive control for these experiments we utilized rat kidney membranes, which

contain both glycosylated high molecular weight, and nonglycosylated monomeric, AQP1 (Agre *et al.*, 1993). Membranes from human embryonic kidney (293) cells exhibited more glycosylated than nonglycosylated hAQP1 after rAAVhAQP1 transduction, while in membranes of similarly treated HSG (human submandibular gland) cells little of the glycosylated hAQP1 was found (Fig. 2, lanes 4 and 8). COS (primate kidney) cells were the only cells studied that endogenously expressed AQP1. After being transduced with rAAVhAQP1, COS cells showed a clear increase in the expression of AQP1 above these endogenous levels (Fig. 2, lane 6).

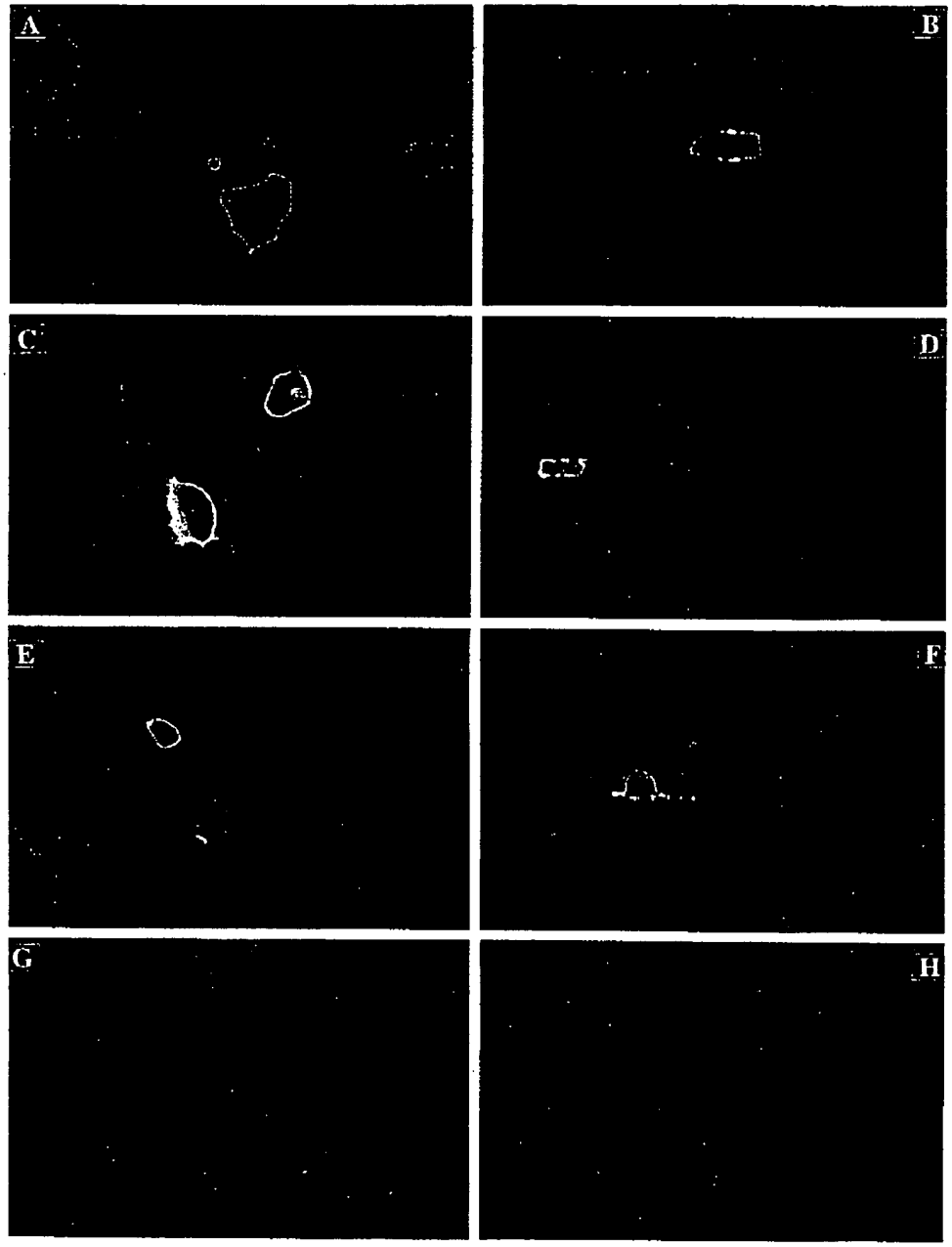

FIG. 3. Localization of hAQP1 in cultured epithelial cells. SMIE (**A** and **B**), HSG (**C** and **D**), and 293 (**E** and **F**) cells were transduced with rAAVhAQP1 at an MOI of 100. hAQP1 protein expression was detected using an affinity-purified antibody and confocal microscopy. The horizontal (**A, C,** and **E**) and vertical (**B, D,** and **F**) views of transduced cells confirm the localization of hAQP1 to the plasma membrane of all cell types. (**G**) and (**H**) represent nontransduced 293 cells immunostained as in (**E**) and (**F**) and showing the absence of hAQP1 endogenous immunoreactivity.

Localization of hAQP1 in transduced cells

Confluent monolayers of SMIE, HSG, and 293 cells, grown on glass coverslips, were next transduced with rAAVhAQP1 (MOI of 100; where as noted above, the MOI is the number of particles per cell and thus each cell was exposed to 0.1 transducing particle). After 24 hr the cellular distribution of hAQP1 was examined by confocal microscopy (Fig. 3). As can be seen for SMIE (Fig. 3A and B), HSG (Fig. 3C and D), and 293 (Fig. 3E and F) cells, only a small fraction of the cells were transduced. Examination in the *xy* (horizontal; Fig. 3A, C, and E) and *xz* (vertical; Fig. 3B, D, and F) planes showed hAQP1 only in the plasma membrane. Figure 3G (*xy* plane) and Fig. 3H (*xz* plane) show results of immunostaining with nontransduced 293 cells.

We also examined the cellular distribution of hAQP1 protein after viral transduction of SMIE cells grown as a polarized monolayer on collagen-coated filters. As shown below, the growth of cells in this manner is useful to assess hAQP1 function *in vitro* (He *et al.*, 1998). Figure 4 displays confocal mi-

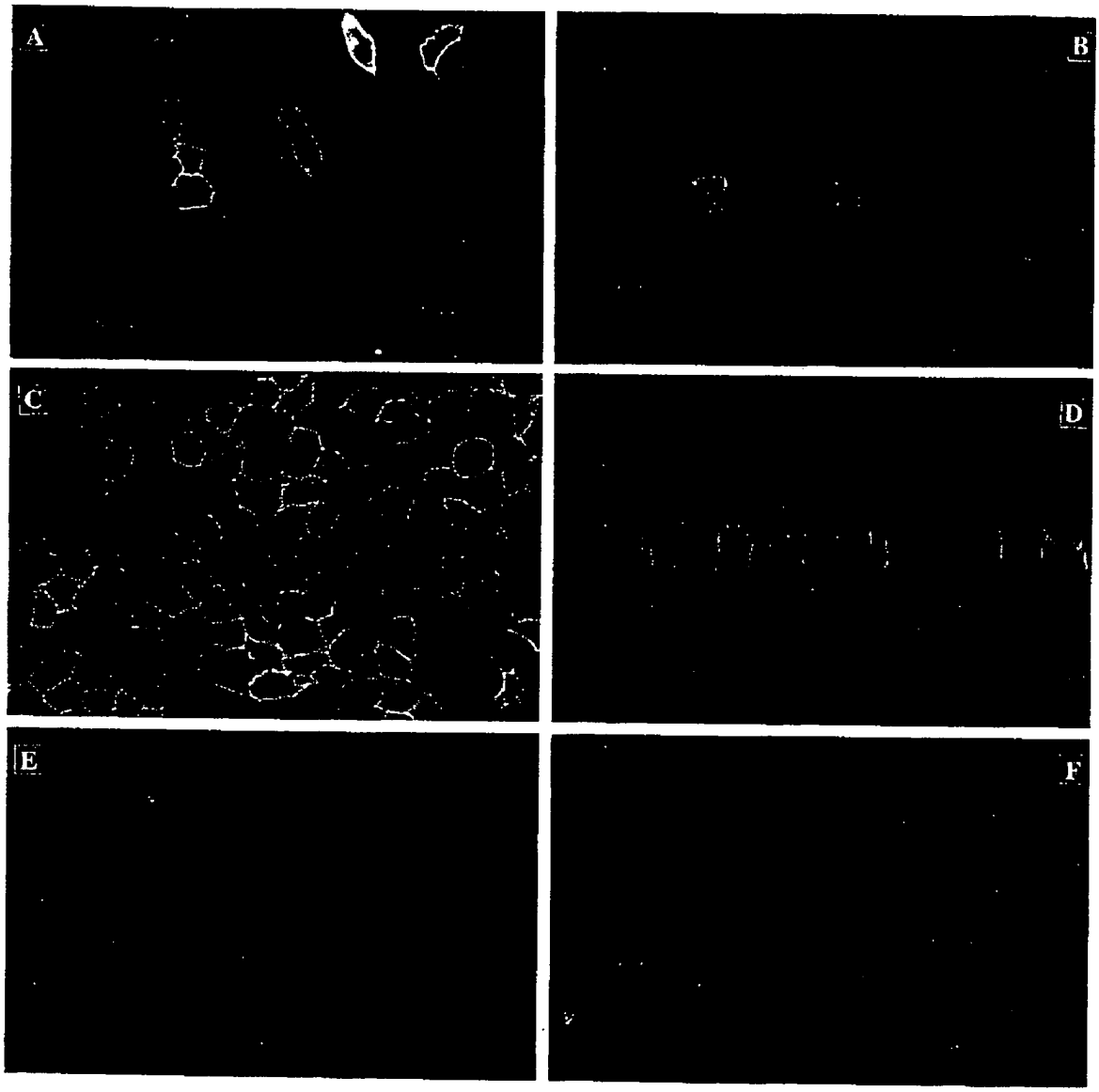

FIG. 4. Localization of hAQP1 in a polarized epithelial monolayer. SMIE cells were grown on collagen-coated filters, transduced or not with rAAVhAQP1 or AdhAQP1, and subjected to an osmotic gradient as described in Fig. 5. Thereafter the monolayers were fixed and stained with antibodies to hAQP1 as described in Materials and Methods. (**A, C,** and **E**) Confocal microscopic images in the horizontal (*xy*) plane; (**B, D,** and **F**) images in the vertical (*xz*) plane. In (**A**) and (**B**) cells were transduced with rAAVhAQP1, while in (**C**) and (**D**) cells were infected with AdhAQP1. (**E** and **F**) Nontransduced cells.

crographs of SMIE cells that are nontransduced (Fig. 4E and F), or transduced with rAAVhAQP1 (Fig. 4A and B) or AdhAQP1 (Fig. 4C and D). As was shown by us earlier (Delporte *et al.*, 1997a, 1998; He *et al.*, 1998), transduction of polarized SMIE cell monolayers by AdhAQP1 at an MOI of 100 (as noted above, for adenoviral transductions MOI is the number of plaque-forming units per cell) leads to uniform infection of cells and the appearance of resultant hAQP1 protein in both the apical and basolateral membranes (Fig. 4C and D). Transduction of monolayers with rAAVhAQP1 (Fig. 4A and B) results in a much smaller proportion of SMIE cells expressing the transgene (~10%). However, all such transduced cells also express the hAQP1 protein in both the apical and basolateral plasma membranes.

Function of recombinant hAQP1 in vitro

We next tested the ability of the recombinant hAQP1 to function *in vitro* (Fig. 5) by measuring fluid movement across a polarized monolayer of SMIE cells. In control cells fluid movement was measured as 75 μl/4 hr in the presence of a transepithelial (apical → basal) osmotic gradient. After transduction of these cells with AdhAQP1, there was a two- to threefold increase in fluid movement. When monolayers were transduced with rAAVhAQP1, fluid movement was also about threefold that in control cells. We also examined fluid movement across cells transduced with rAAVhAQP1 in the presence of wild-type adenovirus and found no differences from that observed with monolayers transduced only with rAAVhAQP1. Two additional control experiments were performed: monolayers were either transduced with an "irrelevant" rAAV (rAAVAP) or with rAAVAP and wild-type adenovirus together. Fluid movement in these two "active" controls was slightly greater than observed with nontransduced cells, but markedly less than seen with cells transduced with rAAVhAQP1.

Expression of hAQP1 in vivo

After determining that the hAQP1 transgene expressed from rAAVhAQP1 was functional and correctly targeted *in vitro*, we examined the ability of this virus to transduce epithelial cells in mice. Since it has been previously shown that murine liver is susceptible to rAAV transduction (e.g., Snyder *et al.*, 1997), we examined the ability of rAAVhAQP1 to transduce mouse hepatic cells. Virus particles (10^8) were delivered to the liver via a tail vein injection. Tissues were harvested 4 weeks after virus administration and frozen sections were examined for expression of hAQP1 with an affinity-purified antibody. Sections of liver from control (nontransduced) mice showed no AQP1-positive immunoreactive staining in hepatocytes (not shown). It was previously reported (Yano *et al.*, 1996; Marinelli and La Russo, 1997) that rat hepatocytes do not express AQP1. Rather, cholangiocytes and endothelial cells are the only cell types in rodent liver that endogenously express AQP1. However, in mice administered rAAVhAQP1 intravenously transduction of hepatocytes was clearly evident (not shown).

In the submandibular glands of nontransduced mice, AQP1-positive staining was seen in endothelial cells of capillaries and venules (Fig. 6A; see also Li *et al.*, 1994). Control mice showed no AQP1-positive staining of their parenchymal cells, similar to results reported earlier for rat salivary glands (Li *et al.*, 1994). When virus particles (10^8) were delivered to submandibular glands via retrograde ductal instillation (Delporte *et al.*, 1997a,b), all glands showed positive AQP1 immunoreactivity in ductal epithelial cells (Fig. 6B). Staining was both membrane bound and cytoplasmic. Interestingly, no consistent immunoreactivity was seen in acinar cells. We also attempted to determine if rAAVhAQP1 could transduce muscle cells as has been reported for other rAAVs (e.g., Xiao *et al.*, 1996; Clark *et al.*, 1997). We were unable to demonstrate unequivocally the transduction of myocytes after injection of the quadriceps muscle, in part because of the large background presence of AQP1 in vascular elements in the sections (not shown). However, using an rAAV encoding β-galactosidase we readily observed the transduction of mouse myocytes (not shown).

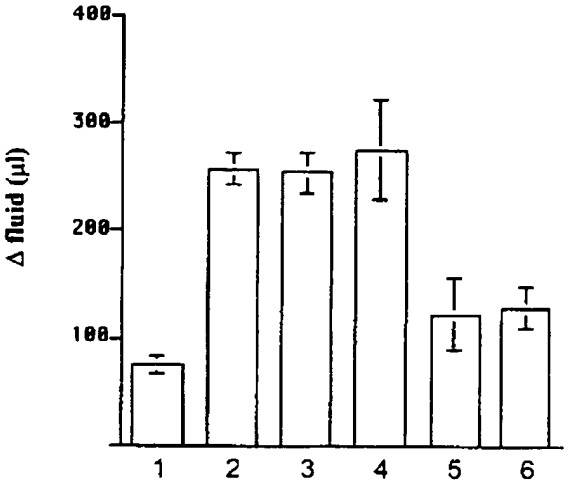

FIG. 5. Net fluid secretion across polarized SMIE cell monolayers. Movement of fluid was measured across intact SMIE cell monolayers in response to an osmotic gradient of 440 mOsm (apical) to 340 mOsm (basal). Net fluid movement in control, nontransduced cells is ~75 μl in 4 hr (1). Fluid movement in cells transduced with AdhAQP1 at an MOI of 100 (2) was about two- to threefold greater. When cells were transduced at an MOI of 100 with rAAV hAQP1 (3), the same level of fluid movement was seen. Level of fluid movement did not significantly change in cells coinfected with rAAVhAQP1 (MOI of 100) and wild-type adenovirus (MOI of 100) (4). No significant fluid movement greater than that found with control, nontransduced cells was seen in cells transduced with rAAVAP alone (5) or when rAAVAP was coinfected with wild-type adenovirus (6). Data represent the mean ± SEM for six separate determinations. Statistical comparisons between various treatment groups were done using either a Student's test or Mann–Whitney *U* test. Differences between groups 1 and 2 ($p < 0.002$), 1 and 3 ($p < 0.001$), and 3 and 5 ($p = 0.003$) were statistically significant, while differences between groups 1 and 5, 2 and 3, 2 and 4, and 5 and 6 were statistically nonsignificant ($p > 0.05$).

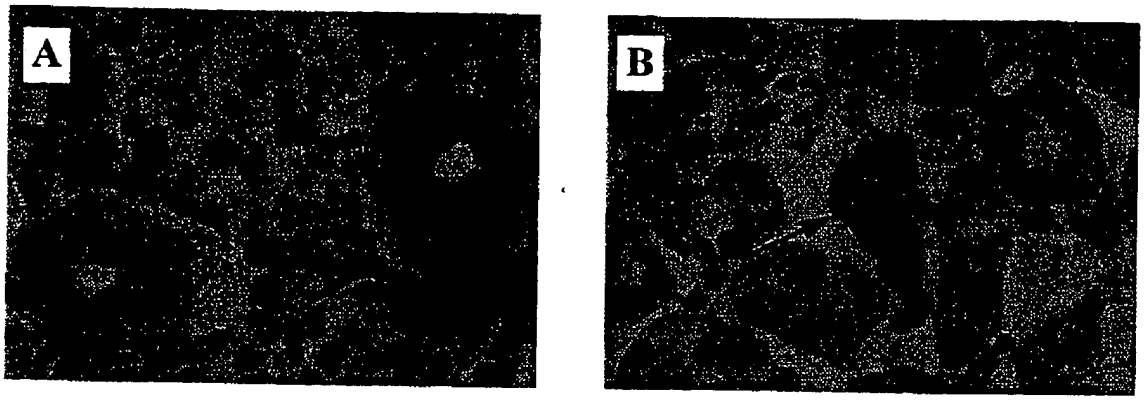

FIG. 6. Detection of AQP1 in frozen sections of mouse submandibular gland. (**A**) Section of submandibular gland from a control, nontransduced animal, depicting background levels of AQP1 in capillaries and venules. (**B**) Section from the corresponding tissue of a mouse treated with 10^8 particles of rAAVhAQP1. Positively stained (reddish-brown) salivary duct cells are indicated by an arrow. Original magnification: ~×160.

DISCUSSION

In the present article we have described the construction of a recombinant AAV, rAAVhAQP1, encoding the human homolog of the archetypal mammalian water channel protein, AQP1. hAQP1 is a plasma membrane-targeted protein found in both the apical and basolateral membrane domains of polarized epithelial and endothelial cells *in vivo* (Agre *et al.*, 1993, 1995). rAAVhAQP1 was produced at relatively high titers. The titers of rAAVhAQP1 achieved were routinely ~10^{11}–10^{12} particles/ml, with a transducing titer in 293 cells of ~10^8–10^9 transducing units/ml. rAAVhAQP1 was capable of transducing four different epithelial cell lines (293, COS, both kidney; HSG, SMIE, both salivary) derived originally from several species: human, monkey, or rat. Interestingly, rAAVhAQP1 was unable to transduce Madin–Darby canine kidney (MDCK) cells, a frequently studied model polarized epithelial cell derived from dog kidney (data not shown). The reason for this latter failure to transduce is not currently understood. A second recombinant AAV, rAAVAP, also was incapable of transducing MDCK cells (not shown). In all transduced epithelial cell lines the recombinant hAQP1 was found in the plasma membrane (Figs. 2–4), exhibiting its natural nonpolarized targeting (Fig. 4). In addition, rAAVhAQP1 was able to transduce mouse salivary ductal epithelial cells and hepatocytes *in vivo*.

Importantly, our *in vitro* studies showed that the hAQP1 protein encoded by rAAVhAQP1 was functional, mediating the transepithelial movement of fluid in response to an osmotic gradient (Fig. 5). The level of fluid movement observed following rAAVhAQP1 transduction was equivalent to that seen when similar polarized SMIE cell monolayers were transduced with AdhAQP1, i.e., about three times control levels. AdhAQP1 resulted in essentially 100% transduction of the target cells, while rAAVhAQP1 led to only 10% of the cells becoming transduced (Fig. 4). Thus, with this convenient *in vitro* model to study fluid movement across an epithelial monolayer (Neufeld *et al.*, 1991; Delporte *et al.*, 1998; He *et al.*, 1998) it appears that submaximal levels of cell transduction are able to impart a maximal physiological response. This finding is consistent with results reported using low MOIs of AdhAQP1 to transduce SMIE cells (Delporte *et al.*, 1998).

In tissue culture, rAAV vectors have been shown to preferentially transduce cells in S phase (about 200-fold relative to nondividing cells; Russell *et al.*, 1994). However, for all studies performed by us, the various epithelial cell lines were either in late-log to early stationary growth (cells grown to near confluence on plastic or glass coverslips) or essentially nondividing (cells grown to a polarized monolayer on collagen-coated filters). In particular, the latter results are significant because salivary epithelial cells *in vivo* are quite polarized and very slowly dividing. In addition, rAAVhAQP1 was capable of transducing the SMIE cell monolayer when the vector was presented to the apical membranes of the cells.

Our *in vivo* studies, showed that mouse salivary glands were susceptible to rAAV transduction. In our experiments, all rAAV-transduced mice studied had detectable levels of hAQP1 expression in their salivary epithelial cells ($n = 3$) and hepatocytes ($n = 3$). Transgene detection in both salivary gland and liver occurred 1 month after rAAV administration. In contrast, adenovirus-mediated AQP expression is close to background levels in rat salivary glands by 7–14 days (Delporte *et al.*, 1997b). Interestingly, we observed a preferential transduction of ductal, versus acinar, cells in the salivary glands. This pattern differs from that seen with recombinant adenoviruses (e.g., Delporte *et al.*, 1997a,b), where both ductal and acinar cells are readily transduced. Our results may reflect an absence or low level of a receptor for AAV binding in murine salivary acinar cell apical membranes (Summerford and Samulski, 1998). Transduced salivary ductal cells exhibited immunopositive hAQP1 in both the cytoplasm and plasma membrane. For the hAQP1 to mediate transcellular water permeability, a plasma membrane localization is necessary. However, as we have shown, relatively little hAQP1 expression in the plasma membrane can mediate considerable transepithelial water flow (Delporte *et al.*, 1998).

As noted earlier, in principle we have demonstrated that it is possible to markedly increase fluid secretion from irradiation-damaged salivary glands *in vivo* (Delporte *et al.*, 1997a).

For such an approach to be truly useful clinically, long-term stable expression of the transgene is desirable. rAAV vectors may offer that possibility, or at least a considerably extended period of expression when compared with adenovirus vectors. Nonintegrated rAAV vectors appear able to survive *in vitro* in stationary cultures (Russell *et al.*, 1994) and rAAVs are capable of integrating into epithelial cell chromosomes, albeit in a non-site-specific manner (Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997). Further, in several tissues, long-term *in vivo* expression of rAAV vectors can occur. These include muscle (Xiao *et al.*, 1996; Clark *et al.*, 1997), lung (Conrad *et al.*, 1996), liver (Snyder *et al.*, 1997), brain (Alexander *et al.*, 1996), and retina (Flannery *et al.*, 1997). Although we do not yet have a complete understanding of the mechanisms involved in the site-specific integration of wild-type AAV (see Kotin, 1994; Urcelay *et al.*, 1995; Chiorini *et al.*, 1996), it is known that the AAV inverted terminal repeats and Rep protein are required. It is reasonably anticipated that such an understanding will one day lead to the development of better vectors for clinical gene therapy. On the basis of the present results, it seems further studies are warranted to examine the utility of rAAV vectors in transducing salivary glands in species phylogenetically closer to humans.

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REFERENCES

ADESANYA, M.R., REDMAN, R.S., BAUM, B.J., and O'CONNELL, B.C. (1996). Immediate inflammatory responses to adenovirus-mediated gene transfer in rat salivary glands. Hum. Gene Ther. **7**, 1085–1093.

AGRE, P., PRESTON, G.M., SMITH, B.L., JUNG, J.S., RAINA, S., MOON, C., GUGGINO, W.B., and NIELSEN, S. (1993). Aquaporin CHIP: The archetypal molecular water channel. Am. J. Physiol. **265**, F463–F476.

AGRE, P., BROWN, D., and NIELSEN, S. (1995). Aquaporin water channels: Unanswered questions and unresolved controversies. Curr. Opin. Cell Biol. **7**, 472–483.

ALEXANDER, I.E., RUSSELL, D.W., SPENCE, A.M., and MILLER, A.D. (1996). Effects of gamma irradiation on the transduction of dividing and nondividing cells in brain and muscle of rats by adeno-associated virus vectors. Hum. Gene Ther. **7**, 841–850.

CARTER, B.J. (1992). Adeno-associated virus vectors. Curr. Opin. Biotech. **3**, 533–539.

CHIORINI, J.A., WENDTNER, C.M., URCELAY, E., SAFER, B., HALLEK, M., and KOTIN, R.M. (1995). High-efficiency transfer of the T cell co-stimulatory molecule B7-2 to lymphoid cells using high titer recombinant AAV vectors. Hum. Gene Ther. **6**, 1531–1541.

CHIORINI, J.A., WIENER, S.M., YANG, L., SMITH, R.H., SAFER, B., KOLCOIN, N.P., LIU, Y., URCELAY, E., and KOTIN, R.M. (1996). The roles of AAV Rep proteins in gene expression and targeted integration. Curr. Top. Microbiol. Immunol. **218**, 25–33.

CLARK, K.R., SFERRA, T.J., and JOHNSON, P.R. (1997). Recombinant adeno-associated viral vectors mediate long-term transgene expression in muscle. Hum. Gene Ther. **8**, 659–669.

CONRAD, C.K., ALLEN, S.S., AFIONE, S.A., REYNOLDS, T.C., BECK, S.E., FEE-MAKI, M., BARRAZZA-ORTIZ, X., ADAMS, R., ASKIN, F.B., CARTER, B.J., GUGGINO, W.B., and FLOTTE, T.R. (1996). Safety of single-dose administration of an adeno-associated virus (AAV)–CFTR vector in the primate lung. Gene Ther. **3**, 658–668.

DELPORTE, C., O'CONNELL, B.C., HE, X., AMBUDKAR, I.S., AGRE, P., and BAUM, B.J. (1996). Adenovirus-mediated expression of aquaporin-5 in epithelial cells. J. Biol. Chem. **271**, 22070–22075.

DELPORTE, C., O'CONNELL, B.C., HE, X., LANCASTER, H.E., O'CONNELL, A.C., AGRE, P., and BAUM, B.J. (1997a). Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. Proc. Natl. Acad. Sci. U.S.A. **94**, 3268–3273.

DELPORTE, C., REDMAN, R.S., and BAUM, B.J. (1997b). Relationship between the cellular distribution of the $\alpha_v\beta_{3/5}$ integrins and adenoviral infection in salivary glands. Lab. Invest. **77**, 167–173.

DELPORTE, C., HOQUE, A.T.M.S., KULAKUSKY, J.A., BRADDON, V.R., GOLDSMITH, C.M., WELLNER, R.B., and BAUM, B.J. (1998). Relationship between adenovirus-mediated aquaporin 1 expression and fluid movement across epithelial cells. Biochem. Biophys. Res. Commun. **246**, 584–588.

DENKER, B.M., SMITH, B.J., KUHAJDA, F.P., and AGRE, P. (1988). Identification, purification, and partial characterization of a novel Mr 28,000 integral membrane protein from erythrocytes and renal tubules. J. Biol. Chem. **263**, 15634–15642.

FLANNERY, J.G., ZOLOTUKHIN, S., VAQUERO, M.I., LA VAIL, M.M., MUZYCZKA, N., and HAUSWIRTH, W.W. (1997). Efficient photoreceptor-targeted gene expression in vivo by recombinant adeno-associated virus. Proc. Natl. Acad. Sci. U.S.A. **94**, 6916–6921.

FLOTTE, T.R., SOLOW, R., OWENS, R.A., AFIONE, S., ZEITLIN, P.L., and CARTER, B.J. (1992). Gene expression from adeno-associated virus vectors in airway epithelial cells. Am. J. Respir. Cell Mol. Biol. **7**, 349–356.

HE, X., FRANK, D.P., and TABAK, L.A. (1990). Establishment and characterization of 12S adenoviral E1A immortalized rat submandibular gland epithelial cells. Biochem. Biophys. Res. Commun. **170**, 336–343.

HE, X., KUIJPERS, G.A.J., GOPING, G., KULAKUSKY, J.A., ZHENG, C., DELPORTE, C., TSE, C.-M., REDMAN, R.S., DONOWITZ, M., POLLARD, H.B., and BAUM, B.J. (1998). A polarized salivary cell monolayer useful for studying transepithelial fluid movement in vitro. Pfluger's Arch. **435**, 375–381.

JOOSS, K., YANG, Y., and WILSON, J.M. (1996). Cyclophosphamide diminishes inflammation and prolongs transgene expression following delivery of adenoviral vectors to mouse liver and lung. Hum. Gene Ther. **7**, 1555–1566.

KAGAMI, H., ATKINSON, J.C., MICHALEK, S.M., HANDELMAN, B., YU, S., BAUM, B.J. and O'CONNELL, B.C. (1998). Repetitive adenovirus administration to the parotid gland: Role of immunological barriers and induction of oral tolerance. Hum. Gene Ther. **9**, 305–313.

KEARNS, W.G., AFIONE, S.A., FULMER, S.B., PANG, M.G., ERIKSON, D., EGAN, M., LANDRUM, M.J., FLOTTE, T.R., and CUTTING, G.R. (1996). Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. Gene Ther. **3**, 748–755.

KESSLER, P.D., PODSAKOFF, G.M., CHEN, X., McQUISTON, S.A., COLOSI, P.C., MATELIS, L.A., KURTZMAN, G.J., and BYRNE, B.J. (1996). Gene delivery to skeletal muscle results in sus-

tained expression and systemic delivery of a therapeutic protein. Proc. Natl. Acad. Sci. U.S.A. **97,** 14082–14087.

KOTIN, R.M. (1994). Prospects for the use of adeno-associated virus as a vector for human gene therapy. Hum. Gene Ther. **5,** 793–801.

KOTIN, R.M., SINISCALCO, M., SAMULSKI, R.J., ZHU, X., HUNTER, L., LAUGHLIN, C.A., McLAUGHLIN, S., MUZYCZKA, N., ROCCHI, M., and BERNS, K.I. (1990). Site-specific integration by adeno-associated virus. Proc. Natl. Acad. Sci. U.S.A. **87,** 2211–2215.

KOZARSKY, K.F., and WILSON, J.M. (1993). Gene therapy: Adenovirus vectors. Curr. Opin. Genet. Dev. **3,** 499–503.

LI, J., NIELSEN, S., DAI, Y., LAZOWSKI, K.W., CHRISTENSEN, E.I., TABAK, L.A., and BAUM, B.J. (1994). Examination of rat salivary glands for the presence of the aquaporin CHIP. Pfluger's Arch. **428,** 455–460.

MACK, C.A., SONG, W.-R., CARPENTER, H., WICKHAM, T.J., KOVEDI, I., HARVEY, B.-G., MAGOVERN, C.J., ISOM, O.W., ROSENGART, T., FALCK-PEDERSEN, E., HACKETT, N.R., CRYSTAL, R.G., and MASTRANGELI, A. (1997). Circumvention of anti-adenoviral neutralizing immunity by administration of an adenoviral vector of an alternate serotype. Hum. Gene Ther. **8,** 99–109.

MARINELLI, R.A., and LA RUSSO, N.F. (1997). Aquaporin water channels in liver: Their significance in bile formation. Hepatology **26,** 1081–1084.

McKEON, C., and SAMULSKI, R.J. (1996). NIDDK workshop on AAV vectors: Gene transfer into quiescent cells. Hum. Gene Ther. **7,** 1615–1619.

McLAUGHLIN, S.L., COLLIS, P., HERMONAT, P.L., and MUZYCZKA, N. (1988). AAV general transduction vectors: analysis of proviral structures. J. Virol. **62,** 1963–1973.

MULLIGAN, R.C. (1993). The basic science of gene therapy. Science **260,** 926–932.

NEUFELD, T.K., GRANT, T.E., and GRANTHAM, J.J. (1991). A method to measure the rate of net fluid secretion by monolayers of cultured renal epithelial cells. J. Tissue Cult. Methods **13,** 229–234.

PONNAZHAGAN, S., ERIKSON, D., KEARNS, W.G., ZHOU, S.Z., NAHREINI, P., WANG, X.-S., and SRIVASTAVA, A. (1997). Lack of site-specific integration of the recombinant adeno-associated virus 2 genomesin human cells. Hum. Gene Ther. **8,** 275–284.

PRESTON, G.M., and AGRE, P. (1991). Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: Member of an ancient channel family. Proc. Natl. Acad. Sci. U.S.A. **88,** 11110–11114.

RUSSELL, D.W., MILLER, A.D., and ALEXANDER, I.E. (1994). Adeno-associated virus vectors preferentially transduce cells in S phase. Proc. Natl. Acad. Sci. U.S.A. **91,** 8915–8919.

SAMULSKI, R.J., CHANG, L.-S., and SHENK, T. (1989). Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J. Virol. **63,** 3822–3828.

SHIRASUNA, K., SATO, M., and MIYAZAKI, T. (1981). A neoplastic epithelial duct cell line established from an irradiated human salivary gland. Cancer **48,** 745–753.

SMITH, T.A.G., WHITE, B.D., GARDNER, J.M., KALEKO, M., and McCLELLAND, A. (1996). Transient immunosuppression permits successful repetitive intravenous administration of an adenovirus vector. Gene Ther. **3,** 496–502.

SNYDER, R.O., MIAO, C.H., PATIJN, G.A., SPRATT, S.K., DANOS, O., DEA, N., GOWN, A.M., WINTHER, B., MEUSE, L., COHEN, L.K., THOMPSON, A.R., and KAY, M.S. (1997). Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nature Genet. **16,** 270–276.

SUMMERFORD, C., and SAMULSKI, R.J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J. Virol. **72,** 1438–1445.

URCELAY, E., WARD, P., WIENER, S.M., SAFER, B., and KOTIN, R.M. (1995). Asymmetric replication in vitro from a human sequence element is dependent on adeno-associated virus Rep protein. J. Virol. **69,** 2038–2046.

WORGALL, S., WOLFF, G., FALCK-PEDERSEN, E., and CRYSTAL, R.G. (1997). Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. Hum. Gene Ther. **8,** 37–44.

XIAO, X., LI, J., and SAMULSKI, R.J. (1996). Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J. Virol. **70,** 8098–8108.

YANG, Y., GREENOUGH, K., and WILSON, J.M. (1996). Transient immune blockade prevents formation of neutralizing antibody to recombinant adenovirus and allows repeated gene transfer to mouse liver. Gene Ther. **3,** 412–420.

YANO, M., MARINELLI, R.A., ROBERTS, S.K., BALAN, V., PHAM, L., TARARA, J.E., DE GROEN, P.C., and LARUSSO, N.F. (1996). Rat hepatocytes transport water mainly via a non-channel-mediated pathway. J. Biol. Chem. **271,** 6702–6707.

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Re-engineered salivary glands are stable endogenous bioreactors for systemic gene therapeutics

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Abbreviations: SG, salivary gland; AAV, adeno-associated virus; LacZ, E. Coli β-galactosidase hEPO, human erythropoietin; LacZ, E. Coli β-galactosidase.

Character Count

Material	Character count
All text characters (including title page, abstract, legends, references) *plus* spaces	31,872
Fig. 1 (1-column, 4 cm high = 180 x 4)	720
Fig. 2 (1-column, 5 cm high = 180 x 5)	900
Fig. 3 (1-column, 5 cm high = 180 x 5)	900
Fig. 4 (1-column, 5 cm high = 180 x 5)	900
Fig. 5 (1-column, 5 cm high = 180 x 5)	900
Fig. 6 (1-column, 5 cm high = 180 x 5)	900
Fig. 7 (1-column, 4 cm high = 180 x 4)	720
Table 1 (1-column, 22 lines high = 60 x 22)	1,320
Space Allowance	
7 single-column figures (7 x 120)	840
1 single-column table (1 x 120)	120
Total characters in paper	40,092

Abstract

The employment of critical-for-life organs (e.g., liver or lung) for systemic gene therapeutics can lead to serious safety concerns. To circumvent such issues, we have considered salivary glands (SGs) as an alternative gene therapeutics target tissue. Given the high secretory abilities of SGs, we hypothesized that administration of low doses of recombinant adeno-associated viral (AAV) vectors would allow for therapeutic levels of transgene-encoded secretory proteins in the bloodstream. We administered 10^9 particles of an AAV vector encoding human erythropoietin (hEPO) directly to individual mouse submandibular SGs. Serum hEPO reached maximum levels 8-12 weeks after gene delivery and remained relatively stable for 54 weeks (longest time studied). Hematocrit (Hct) levels were similarly increased. Moreover, these effects proved to be vector dose dependent, and even a dosage as low as 10^8 particles/animal led to significant increases in hEPO and Hct levels. Vector DNA was only detected within the targeted SGs and levels of AAV copies within SGs were highly correlated with serum hEPO levels ($r=0.98$). These results show that SGs appear to be promising targets, with potential clinical applicability, for systemic gene therapeutics.

Introduction

Gene therapeutics, use of the gene as a drug, was initially proposed as a highly promising clinical application of gene transfer (1,2). Currently, most therapeutic transgenes intended for correction of systemic single protein deficiency disorders, are delivered to either a major, critical-for-life organ, e.g., liver or lung (3,4), or a tissue not physiologically intended for secretion, e.g., muscle (5,6). Both approaches although useful can be unsatisfactory, since they can require relatively high vector doses to achieve effective therapy (3-6). Furthermore, targeting a critical organ, such as the liver or lung, can lead to serious safety concerns (7,8). An ideal target tissue for gene therapeutics should be readily accessed, ensure safety, and lead to adequate as well as stable transgenic protein production.

The quest for a better gene therapeutics target tissue led us to consider an unusual candidate: the salivary glands (SGs). Human SGs consist of extremely active secretory cells, able to produce 0.75-1.5 liters of saliva daily, as well as to secrete considerable amounts of protein into both the gastrointestinal tract and bloodstream (9). Importantly, SGs are well encapsulated, not critical-for-life organs. Therefore, it seemed to us reasonable to try to take advantage of their potential utility for gene therapeutics.

Gene transfer to SGs is accomplished in a relatively non-invasive manner by intra-oral cannulation of the main excretory ducts (10). In earlier experiments, we used recombinant serotype 5 adenoviral vectors for gene transfer and demonstrated successful vector administration, and subsequent protein production and secretion of several therapeutic proteins, albeit for a limited time (e.g., 11-13). Very recently, we demonstrated that adeno-associated virus (AAV) vectors can be used for gene transfer to

SGs (14). In the present study, we employ AAV vectors in SGs in an attempt to circumvent some important existing obstacles to successful gene therapeutics. Specifically, we evaluated the hypothesis that the administration of low doses of AAV vectors to SGs can lead to the stable, long-term secretion of a therapeutic protein into the bloodstream for systemic disease applications. Our experiments clearly demonstrate proof of this hypothesis and strongly support the notion that SGs may be valuable gene transfer targets for gene therapeutics with potential clinical significance.

Materials and Methods

Construction f AAVhEPO and AAVLacZ

Generation of AAV vectors was performed as previously described (14-16). Briefly, 293T cells were co-transfected with the *trans* plasmids pMMTV2.1 (provides the *Rep* and *Cap* genes), pAd12 (provides adenoviral helper genes) and the *cis* plasmid containing either the E. Coli β-galactosidase (LacZ) or human erythropoietin (hEPO) cDNA flanked by the AAV ITRs (Figure 1) at a ratio of 1:1:1 by calcium phosphate precipitation to generate the recombinant vector AAVLacZ or AAVhEPO, respectively. Transgene expression in both recombinant AAV vectors was driven by the Rous Sarcoma Virus (RSV) promoter. Dr. Y. Terada (Tokyo Medical and Dental University) generously provided the hEPO cDNA. The cells were harvested 48 hr post transfection and a crude viral lysate (CVL) obtained after three freeze-thaw cycles. The lysate was treated with benzonase (100U/mL of CVL; 37°C for 45min), adjusted to a refractive index of 1.372 by addition of CsCl and centrifuged at 38,000 rpm for 65 hr at 20°C. Equilibrium density gradients were fractionated and fractions with a refractive index of 1.369-1.375 were collected and stored at 4°C and assayed for infectious activity. The number of AAV genomes was estimated using real time quantitative (Q) PCR (Applied Biosystems, Foster City, CA; see below). Immediately before experiments, viral fractions were dialyzed against 0.9% NaCl.

Mice, gene transfer, saliva and serum collections

Animal studies were approved by the NIDCR Animal Care and Use Committee and the NIH Biosafety Committee. All procedures were conducted in accordance with IASP

standards. Male Balb/c mice were obtained from the Division of Cancer Treatment, NCI, Bethesda, MD. Mice (four groups, n=5 each) were administered 10^9 particles (suspended in 50µl of 0.9% NaCl) of either AAVLacZ (n=10) or AAVhEPO (n=10) by retrograde ductal delivery to their submandibular SGs (10,14). Two additional groups of Balb/c mice (n=5 each) were also utilized for a vector dose-response evaluation, and received 10^8 or $5x10^9$ AAVhEPO particles, respectively. A further group (n=5) of naïve mice (administered with 50µl of 0.9% NaCl) was included. Mild anesthesia was induced with 1 µl/g body weight of a 60 mg/ml ketamine (Phoenix Scientific, St. Joseph, MO) and 8 mg/ml xylazine (Phoenix Scientific) solution given intramuscularly. One cohort of mice, administered either AAVLacZ (n=5) or AAVhEPO (n=4; one animal died), and the naïve group (n=4), were sacrificed 8 weeks after viral administration. Blood and tissue (submandibular glands, liver, spleen and testis) samples were collected. The other mice (AAVLacZ and AAVhEPO, n=5 each) were maintained throughout the experiment (up to 54 weeks). Blood samples were obtained by orbital bleeding. Whole saliva was collected as described (14), after stimulation of secretion using 0.5 mg pilocarpine/kg body weight administered subcutaneously. Hematocrits (Hcts) were determined using micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA).

Quantification of hEPO

Secretion of hEPO in mouse serum and saliva was determined by an ELISA using commercial assay kits (R&D systems, MN, USA). The lower limit of detection was 0.6 mU/ml. Assays were performed according to the manufacturer's instructions.

Quantitative real-time PCR

Genomic DNA was isolated from submandibular glands, liver, spleen and testes of treated and untreated mice (week 8) using the DNeasy isolation kit (Qiagen, Chatsworth, CA). QPCR amplification (20 µl final volume) of the DNA (100 ng) was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) by using the SYBR Green PCR Master Mix (Applied Biosystems) and a specific 5' and 3' primer pair appropriate (0.3 µM; 5': GATGAGTTAGCAACATGCCTTACAA, 3': TCGTACCACCTTACTTCCACCAA) for the RSV promotor. A PCR thermal profile of holding at 95°C for 10 min, denaturing at 95°C for 15 s, and annealing and extending for 60°C for 1 min was performed for 40 cycles. A standard curve, employing the AAVEPO plasmid and including 100 ng genomic DNA of untreated animals for each specific tissue, was included for each QPCR reaction. Triplicate samples were assayed in a single run.

Histologic assessment of submandibular glands

Submandibular glands were removed for histologic analyses from AAVLacZ treated mice (n=5) at the time of sacrifice (week 8), embedded in medium for frozen tissue specimens (O.C.T. Compound, Sakura Finetek, Torrance, CA) and placed on dry ice. Sections were cut at 5 µm thickness. Infected cells were detected by immunohistochemical analyses (β-galactosidase positive nuclear staining) using the streptavidin-biotin peroxidase complex method. Frozen sections from animals administered AAVEPO served as controls for the β-galactosidase staining experiment. Briefly, after endogenous peroxidase, streptavidin and biotin activities were blocked

(Streptavidin/Biotin Blocking Kit SP-2002, Vector Laboratories; Burlingame, CA), sections were incubated overnight at 4°C with a polyclonal primary antibody against β-galactosidase (B59136; Biodesign, Saco, Maine; 1:500) raised in rabbits. Staining was developed by using a biotinylated goat antibody (Vector Laboratories) directed against the primary antibody and the avidin-biotin peroxidase complex followed by 3,3'-diaminobenzidine (SK-4100; Vector Laboratories) and counterstained with hematoxylin.

Southern Hybridization

Hirt extracted DNA, from submandibular glands of both treated and naïve animals (week 8), was used in the Southern hybridization analyses (17). Briefly, tissue samples were incubated in Hirt buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 1% SDS, 10 μg of DNase-free RNase and 0.5 mg/ml proteinase K) at 37°C overnight. NaCl was added to the digestion mixture at a final concentration of 1.0 M. After overnight incubation at 4°C, samples were centrifuged at 8000 x *g* for 20 min. The supernatant, containing low molecular weight DNA, was phenol-chloroform extracted, ethanol precipitated and the DNA dissolved in TE buffer. The pellet, containing high molecular weight genomic DNA, was washed several times with 75% ethanol and finally dissolved in TE buffer. Low molecular weight DNA was either undigested or digested with *Sma* I, *BssH* II or Plasmid Safe DNase (EPICENTRE, Madison,WI), an enzyme cutting linear DNA forms (18), while high molecular weight DNA was digested with *Sma* I. DNA from each sample (30 μg), was separated on 1% agarose gels, transferred to Nylon membranes and hybridized with an [α-^{32}P]dCTP-radiolabeled RSV-EPO probe (an 1264-bp Sma I fragment from the AAVEPO plasmid; Figure 1).

Results

Serum hEPO and Hct levels

Mean serum hEPO levels were non-detectable and mean Hct levels (±SE) were 60.2 ± 2 % prior to administration (week 0), for all animals used in our experiments. In the AAVhEPO treated group (10^9 particles/animal; n=5) serum hEPO levels gradually increased for a 12 week period (Figure 2; mean ± SE: 27.1 ± 13.9 mU/mL) and remained relatively stable thereafter with respect to each individual mouse (Figure 3; week 54; longest time studied). For example, on week 28, serum hEPO levels avereged 20.2 ± 12 mU/ml, while on week 48, serum hEPO levels avereged 19.5 ± 10.1 mU/mL (Figure 2). Hct levels increased in parallel with the serum hEPO levels (84.4 ± 4 % on week 12), and remained elevated up until the end of the experiment (80 ± 4.8 and 81.2 ± 4.5 on weeks 28 and 48, respectively; Figure 2). Serum hEPO levels in the AAVLacZ administered group (control group) remained undetectable throughout the 54 weeks of the experiment (data not shown). No change was observed in the Hct levels in the control group which remained ~60% up until the end of the experiment (data not shown). The levels of hEPO detected in saliva were minimal (0.6 ± 0.6 mU/mL; n=5) in mice treated with AAVhEPO. Serum hEPO levels in the other two dosage groups tested (n=5 each; receiving either 10^8 or $5x10^9$ particles/animal) gradually increased from non-detectable on week 0 to 7.8±3.8 mU/ml (n=3) and 60.5±42.1 mU/ml (n=4) on week 12, respectively (Figure 4). Hct levels in the same groups on week 12 were 72.3±7.2 and 87.7±2.5%, respectively.

Three groups of mice were sacrificed on week 8 of these experiments. In the group administered AAVhEPO (n=4, 10^9 particles/animal), mean serum hEPO levels were 15.2±5.1 mU/ml (Hct: 77.5±5.2). Conversely, serum hEPO levels remained non-

detectable in both the AAVLacZ (n=5, 10^9 particles/animal) and naïve (n=4; 0.9% NaCl) groups, as observed on week 0 (data not shown).

Quantitative real-time PCR (QPCR)

QPCR was performed, using DNA extracted from submandibular glands, liver, spleen and testis, to evaluate the number of viral copies present. Samples were obtained from the AAVhEPO group (n=4; 10^9 particles/animal) and the naïve animal group (n=4; 0.9% NaCl), sacrificed on week 8. QPCR results are depicted in Table 1. Viral DNA was only present in the SGs of AAVhEPO treated animals (13185±2963 copies/100ng of extracted DNA; n = 4). Naïve animals yielded background levels of 480±32 copies/100ng of DNA extracted from their SGs. There were no differences detected in the viral DNA present in the liver, spleen and testis between AAVhEPO treated and naïve animals. Furthermore, the correlation between viral copies present in the SGs of the AAVhEPO treated animals and serum hEPO levels was highly significant (r=0.98; Figure 5).

Histologic assessment of submandibular glands

Immunocytochemistry assays were performed on SG tissue sections from AAVLacZ (n=5; 10^9 particles/animal) and AAVhEPO (n=4; 10^9 particles/animal) treated animals, sacrificed on week 8 (Figure 6). β-galactosidase expression was only observed in the AAVLacZ treated group. The AAVs infected 10-15% of the SG cells. Only ductal cells had positive nuclear staining (no staining was observed in the acinar cells). Additionally, no inflammatory infiltrate or structural abnormality was observed in the gland sections from mice treated with AAV vectors. No nuclear staining was observed in the tissue

sections obtained from AAVhEPO treated animals, using the same immunocytochemistry procedure (Figure 6 inset).

Southern Hybridization

Southern hybridization analysis was performed in order to examine the form of viral DNA present in SGs (Figure 7). Hirt extracted DNA, either undigested or digested with either *Sma* I, *BssH* II, or Plasmid Safe DNase, was examined. DNA was obtained from submandibular glands of both AAVhEPO treated (n=4; 10^9 particles/animal) and naïve animals (n=4; 0.9% NaCl) sacrificed on week 8. Plasmid Safe DNase only cuts linear forms of DNA, circular forms remaining intact (18). Undigested low molecular weight DNA from the AAVhEPO treated group showed two hybridization positive bands (Figure 7, lane a). The larger band corresponded to the length of the recombinant AAVhEPO viral DNA (1958 bp). Digestion of the same DNA, with *Sma* I or *BssH* II resulted in 1264 bp and 1740 bp fragments, respectively (Figure 7, lane b and c). These fragments correspond to those resulting from digestion of the AAVhEPO plasmid with the same enzymes (data not shown; see AAVhEPO construct in Figure 1). A smaller band was also observed in lane c. This band corresponded to the lower band in lane a, but was significantly weaker. After digestion of the extracted DNA with Plasmid Safe only one band remained corresponding with the lower band in the undigested DNA lane (Figure 7, lanes a and d). When high molecular DNA (extracted from the same SGs) was used after digestion with *Sma* I, no hybridization positive bands were observed (data not shown). In addition, no hybridization positive bands were observed in the Southern blots when DNA extracted from naïve animals was used (data not shown).

Discussion

The use of a major, critical-for-life organ, e.g., liver or lung (3,4), or a tissue not physiologically intended for secretion, e.g., muscle (5,6), as targets for systemic gene therapeutics, although useful, can present certain obstacles with respect to clinical applicability. For example, use of the former can potentially lead to serious safety issues (7,8), while use of the latter may not provide adequate levels of therapeutic protein production at low vector doses (19,20). SGs are self-confined (encapsulated), non-critical for life organs with striking secretory abilities. These features motivated us to suggest SGs as potential targets for gene therapeutics (11). Existing data suggest that SGs could be excellent targets for gene transfer with respect to safety and protein productivity (see 10-13, 21). Moreover, in case of an unanticipated severe adverse event following vector delivery, a single SG can be removed surgically (in contrast to liver or lung) with relatively little morbidity. The fact that AAV vectors can be successfully used to stably deliver transgenes to the SGs is an additional safety consideration, since there is no evidence that AAV is an etiological agent for human disease (22). To further ensure safety, the use of SG tissue-specific promoters can be beneficial, as it would preclude transgene production from other tissues (23).

SG cells exhibit at least two distinct secretory pathways: a predominant regulated one leading to exocrine protein secretion into saliva via zymogen granules and a constitutive one leading to the bloodstream (9,13,24). Secretory proteins produced as transgene products in SGs, after viral-mediated gene transfer, continue to follow the same general secretory pathway as in their primary site of production (e.g. α1 anti-trypsin follows the constitutive pathway and growth hormone follows the regulated pathway;

11,13, 24). EPO is secreted primarily from kidney tubular epithelial cells (25) via a constitutive pathway, targeting erythroid progenitor cells in the bone marrow and increasing red blood cell production and therefore Hct levels.

As we hypothesized, hEPO produced in mouse submandibular SGs was preferentially secreted into the bloodstream (constitutive pathway) throughout the entire length of our experiments, with salivary hEPO levels being minimal. Mean serum hEPO levels in the mice treated with AAVhEPO steadily increased for an 8-12 week period and remained relatively stable thereafter (week 48; Figure 2). The stability of serum hEPO levels was even more apparent within individual mice (week 54, longest time studied; Figure 3). Differences observed between individual mice are likely a result of both animal variability, and variability in the effectiveness of cannulation of SGs in 20g mice. This latter problem is not a concern in larger animals and humans (10, 26). Notably, SGs were able to sustain serum hEPO levels well within the normal range (10-30 mU/mL; 27). In addition, circulating hEPO proved to be not only therapeutically adequate but also functional throughout the experiment: Hct levels in all AAVhEPO treated groups increased in parallel with serum hEPO levels. This indicates that SGs are capable of all the necessary post-transcriptional modifications required for the secretion and biological activity of this transgene product (28). Importantly, serum hEPO levels and Hct values were unaffected in the AAVLacZ treated group.

SGs are directly accessed, in a relatively non-invasive manner, such as is performed clinically without anesthesia for contrast radiographs (10). During the procedure, almost the entire SG epithelial cell population is directly accessible to vector suspended in a small volume (50μl for mice) within a confined space, and thus is a

potential gene transfer target (10). The latter circumstance along with the considerable physiological ability of SG cells to produce, process and secrete high levels of proteins, minimizes the amount of vector needed for AAV-mediated gene transfer with SGs. As a result, the vector dose (10^9 particles/animal) used in most of our experiments was 10-100 fold lower than typically reported with liver, lung and muscle (3-6,19,20,29). Furthermore, even a dose as low as 10^8 particles/animal led to clearly measurable and effective EPO levels in the bloodstream (Figure 4), indicating that the considerable protein production and secretory mechanisms in the SGs were easily "exploited".

We used real-time QPCR to measure viral load in SGs, testes, liver and spleen from both treated and naïve animals. Viral DNA was only detected in the targeted SGs (13185±2963 copies/100ng of extracted DNA; n = 4). Thus, undesirable dissemination of the virus beyond the SGs was below the sensitivity level of the assay, and administered AAV vector appears confined within the glands (Table 1). These results likely are attributable both to the encapsulation of SGs, as well as the small volumes of infusate and low viral doses used in our experiments.

We also considered whether the SGs were the actual site of hEPO production observed. We conclude that the QPCR results strongly indicate that the hEPO measured in serum was solely produced by and secreted from the targeted SGs. As noted, viral DNA was essentially only detected in the targeted SGs. DNA tested from liver, spleen and testis showed a similar number of viral copies in the QPCR analysis as measured in these tissues from naïve mice. Additional support for this conclusion is derived from the fact that the number of viral copies present in the SGs and the serum hEPO levels were strongly correlated (r=0.98; Figure 5).

DNA was also extracted from SGs and examined in an attempt to study the fate of the recombinant AAV DNA after administration. Southern hybridization analyses suggest that viral DNA was present as both double-stranded linear and stable circular forms (Figure 7). The formation of these structures appears to be a key event for the AAV-mediated stable production of transgene products (30). As with results in muscle tissue (31), the primary form of AAV in the SGs appears to be episomal.

Immunocytochemistry assays performed on SG tissue sections from AAVLacZ treated animals demonstrated that the AAVs infected 10-15% of the SG cells (Figure 6). Only ductal cells appeared susceptible to AAV infection. Acinar cells probably lack either appropriate viral receptors or key intracellular components for successful transduction. Importantly, no inflammatory infiltrate or structural abnormality was observed in gland sections at 8 weeks after administration, suggesting that retrograde ductal delivery of AAVs does not cause any irreversible damage to the SG tissue.

Interestingly, the life span of salivary epithelial cells in rodents has been estimated to be 125-200 days (32,33), but the ability of SGs cells to divide is a controversial issue (34,35). Our data suggest that either the transduced salivary ductal cells do not significantly divide during the 54-week period studied, in contrast to previous life span estimates in rodents, or that if cell division occurs, transduced daughter cells remain functionally active within the SG tissue.

In summary, we have hypothesized that the use of SGs for systemic gene therapeutics is consistent with the primary biological role of this tissue, i.e. utilizing normal protein production and secretion pathways. Our results show that stable, long-term production of therapeutic serum levels of a functional protein can indeed be

achieved relatively non-invasively in SGs by low doses of AAV vectors. Thus, SGs appear able to act as self-contained, endogenous bioreactors following gene transfer and are therefore unusual yet promising targets for systemic gene therapeutics.

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References

1. Felgner, P.L., & Rhodes, G. (1991) *Nature* **349**, 351-352.

2. Crystal, R.G. (1995) *Nat. Med.* **1**, 15-17.

3. Snyder, R.O. ,Miao, C.H., Patijn G.A., Spratt, S.K., Danos, O., Nagy, D., Gown, A.M., Winther, B., Meuse, L., Cohen, L.K., *et al.* (1997) *Nat. Genet.* **16**, 270-276.

4. Auricchio, A., O'Connor, E., Weiner, D., Gao, G.P., Hildinger, M., Wang, L., Calcedo, R., & Wilson, J.M. (2002) *J. Clin. Invest.* **110**, 499-504.

5. Johnston, J., Tazelaar, J., Rivera, V.M., Clackson, T., Gao, G.P., & Wilson, J.M. (2003) *Mol. Ther.* **7**, 493-497.

6. Bohl, D., Bosch, A., Cardona, A., Salvetti, A., & Heard, J.M. (2000) *Blood* **95**, 2793-2798.

7. Carmen, I.H. (2001) *Mol. Ther.* **3**, 425-428.

8. Marshall, E. (2000) *Science* **288**, 951-957.

9. Isenman, L., Liebow, C., & Rothman, S. (1999) *Am. J. Physiol.* **276**, E223-E232.

10. Baum, B.J., Wellner, R.B., & Zheng, C. (2002) *Int. Rev. Cytol.* **213**, 93-146.

11. Kagami, H., O'Connell, B.C., & Baum, B.J. (1996) *Hum. Gene Ther.* **7**, 2177-2184.

12. He, X., Goldsmith, C.M., Marmary, Y., Wellner, R.B., Parlow, A.F., Nieman, L.K., & Baum, B.J. (1998) *Gene Ther.* **5**, 537-541.

13. Baum, B.J, Berkman, M.E., Marmary, Y., Goldsmith, C.M., Baccaglini, L., Wang, S., Wellner, R.B., Hoque, A.T., Atkinson, J.C., Yamagishi, H., *et al.* (1999) *Hum. Gene Ther.* **10**, 2789-2797.

14. Yamano, S., Huang, L.Y., Ding, C., Chiorini, J.A., Goldsmith, C.M., Wellner, R.B,, Golding, B., Kotin, R.M., Scott, D.E., & Baum, B.J. (2002) *Hum. Gene Ther.* **13**, 287-298.

15. Chiorini, J.A., Wendtner, C.M., Urcelay, E., Safer, B., Hallek, M., & Kotin, R.M. (1995) *Hum. Gene Ther.* **6**, 1531-1541.

16. Kaludov, N., Handelman, B., & Chiorini, J.A. (2002) *Hum. Gene Ther.* **13**, 1235-1243.

17. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365-369.

18. Almazan, F., Gonzalez, J.M., Penzes, Z., Izeta, A., Calvo, E., Plana-Duran, J., & Enjuanes, L. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 5516-5521.

19. Chao, H., Monahan, P.E., Liu, Y., Samulski, R.J., & Walsh, C.E. (2001) *Mol. Ther.* **4**, 217-222.

20. Kessler, P.D., Podsakoff, G.M., Chen, X., McQuiston, S.A., Colosi, P.C., Matelis, L.A., Kurtzman, G.J., & Byrne, B.J. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14082-14087.

21. O'Connell, B.C., Zheng, C., Jacobson-Kram, D., & Baum, B.J. (2003) *J. Oral Pathol. Med.* **32**, 414-421.

22. Kay, M.A., Glorioso, J.C., & Naldini, L. (2001) *Nat. Med.* **7**, 33-40.

23. Zheng, C., Hoque, A.T., Braddon, V.R., Baum, B.J., & O'Connell, B.C. (2001) *Hum. Gene Ther.* **12**, 2215-2223.

24. Hoque, A.T., Baccaglini, L., & Baum, B.J. (2001) *Hum. Gene Ther.* **2**, 1333-1341.

25. Mujais, S.K., Beru, N., Pullman, T.N., & Goldwasser, E. (1999) *Cell Biochem. Biophys.* **30**, 153-166.

26. Li, J., Zheng, C., Zhang, X., Liu, X., Zhang, C., Goldsmith, C.M., Baum, B.J., & Wang, S. *J. Gene Med.* (in press).

27. Kendall, R.G. (2001) *Clin. Lab. Haem.* **23**, 71-80.

28. Dube, S., Fisher, J.W., & Powell, J.S. (1988) *J. Biol. Chem.* **263**, 17516-17521.

29. Samakoglu, S., Bohl, D., & Heard, J.M. (2002) *Mol. Ther.* **6**, 793-803.

30. Duan, D., Sharma, P., Yang, J., Yue, Y., Dudus, L., Zhang, Y., Fisher, K.J., & Engelhardt, J.F. (1998) *J. Virol.* **72**, 8568-8577.

31. Schnepp, B.C., Clark, K.R., Klemanski, D.L., Pacak, C.A. & Johnson, P.R. (2003) *J. Virol.* **77**, 3495-3504.

32. Zajicek, G., Yagil, C., & Michaeli, Y. (1985). *Anat Rec.* **213**, 150-158.

33. Cameron, I.L. (1970) *Tex. Rep. Biol. Med.* **28**, 203-248.

34. Denny, P.C., Ball, W.D., & Redman, R.S. (1997) *Crit. Rev. Oral Biol. Med.* **8**, 51-75.

35. Redman RS. (1995) *Anat. Rec.* **241**, 529-540.

Table 1. Number of viral c pies in tissues after AAV vector administrati n to mouse submandibular glands *

Tissue	AAVhEPO	Control
SALIVARY GLANDS	13185	480
SPLEEN	336	303
LIVER	567	511
TESTIS	183	204

*AAVhEPO (10^9 particles/animal suspended in 50µl of 0.9% NaCl) was delivered to mouse submandibular glands via intra-ductal cannulation. Control (naïve) animals were administered 50µl of 0.9% NaCl, via the same route. After 8 weeks, animals (n=4/group) were euthanized, the indicated tissues obtained, and DNA extracted as described in Materials and Methods. Viral copy number was determined by QPCR as described in Materials and Methods.

Legends

Fig. 1. AAVhEPO construct. The vector AAVhEPO was constructed as described in Materials and Methods. Inverted Terminal Repeat (ITR), Rous Sarcoma Virus promoter (RSV), human erythropoietin cDNA (hEPO) and polyadenylation signal (Poly-A) are shown. Arrows indicate restriction enzyme sites: *Sma* I (⟶) and *BssH* II (⟶). Black line (•⟶•) indicates the probe used in the Southern Hybridization Blot (see Figure 7). Red and blue lines connecting arrows indicate the DNA fragments observed in the Southern Hybridization Blot after digestion with the specific enzymes.

Fig. 2 Serum erythropoietin and hematocrit levels in AAVhEPO treated mice. Vector (AAVhEPO; 10^9 particles/animal) was administered to Balb/c mouse submandibular salivary glands (n=5) by retrograde ductal delivery as described in Materials and Methods. Data shown are the mean (+SE) serum hEPO (blue line, left y axis) and Hct (red line, right y axis) levels measured over a 48 week period.

Fig. 3 Serum hEPO levels in three individual mice after AAVhEPO administration. Vector (10^9 particles/animal) was delivered as in Figure 2, and hEPO was measured over a 54 week period (longest time studied; * animal died or sacrificed at indicated time points).

Fig. 4 hEPO expression is viral vector dose dependent. Three groups of Balb/c mice received either 10^8, 10^9, or 5 x 10^9 particles/animal of the AAVhEPO vector via retrograde ductal cannulation of the submandibular salivary glands as described in Figure 2. Bars represent mean (+ SE) serum hEPO levels 12 weeks after vector administration for each dosage group (n=3, 5 and 4 animals/group, respectively).

Fig. 5 Relationship between hEPO expression in serum and viral copies present in salivary glands in treated and naïve animals. Mice were sacrificed 8 weeks after administration (n=4; 10^9 particles of AAVhEPO/treated animal or 50µl 0.9% normal saline/naïve animal) and viral copies present in the SGs, and serum hEPO levels, were measured as described in Materials and Methods. The correlation between viral copies present and serum hEPO levels was highly significant (r=0.98). Red (*) indicates the mean hEPO serum levels and mean number of viral copies present in salivary glands of naïve animals (n=4).

Fig. 6 Immunocytochemical detection of β-galactosidase expression in mouse submandibular glands. Cryosections were prepared 8 weeks after AAVLacZ administration to mouse submandibular SGs (n=5, 10^9 particles/animal), and β-galactosidase expression detected using an anti-β-galactosidase antibody. Sections were counterstained with hematoxylin. The β-galactosidase cDNA used contains a nuclear localization signal. Red arrows indicate representative cells with nuclear localized β-galactosidase. Staining was observed only in salivary ductal cells. No staining was

detected in the control cryosections obtained from an animal receiving AAVhEPO using the same immunocytochemistry procedure, as shown in the right upper inset.

Fig. 7 Southern Hybridization Blot of low molecular weight DNA from the salivary glands of AAVhEPO treated animals. Vector (10^9 particles/animal) was administered and Hirt extracted DNA prepared as described in Materials and Methods. Undigested Hirt extracted DNA showed that the recombinant AAVhEPO viral DNA is 1958 bp (lane a). Digestion of the Hirt extracted DNA with *Sma* I (lane b) and *BssH* II (lane c) resulted in 1264 bp and 1740 bp fragments respectively, as expected (Figure 1). Blue and red colors correspond with the red and blue lines in Figure 1 indicating the restriction enzyme sites and the resulting DNA fragments. Digestion with Plasmid Safe (an enzyme only cutting linear DNA forms) indicated the presence of episomally maintained circular forms (lane d). Hirt extracted DNA from naïve animals yielded no hybridization positive bands on Southern analyses (data not shown).

Figures

Figure 1

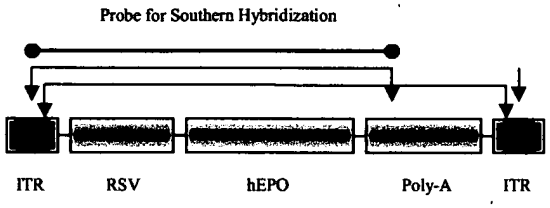


Figure 2

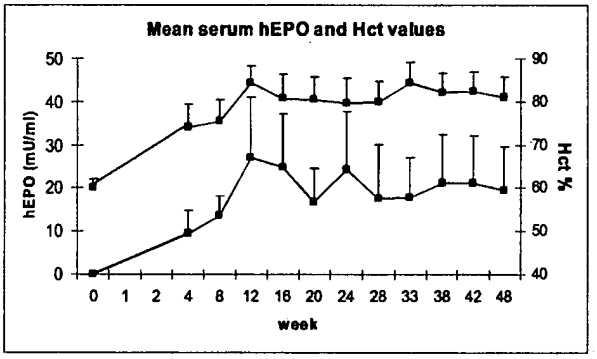


Figure 3

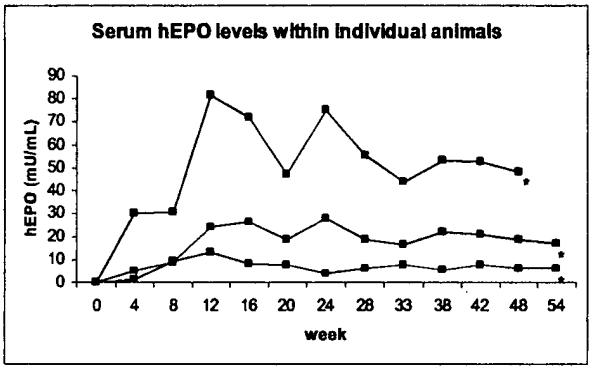


Figure 4

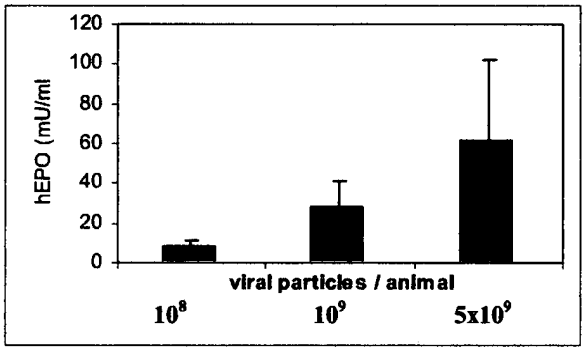


Figure 5

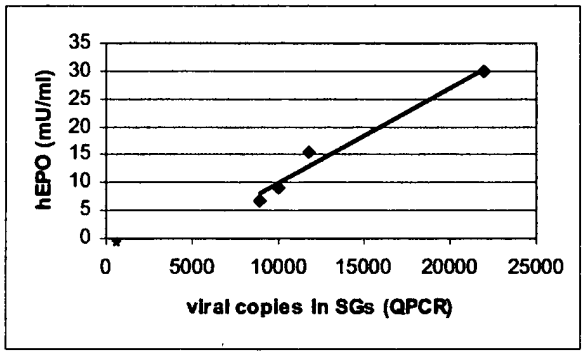


Figure 6

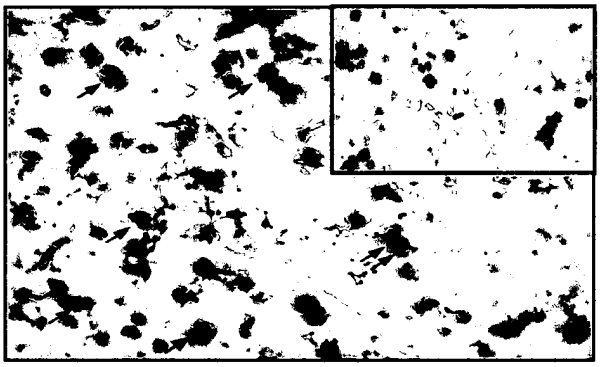
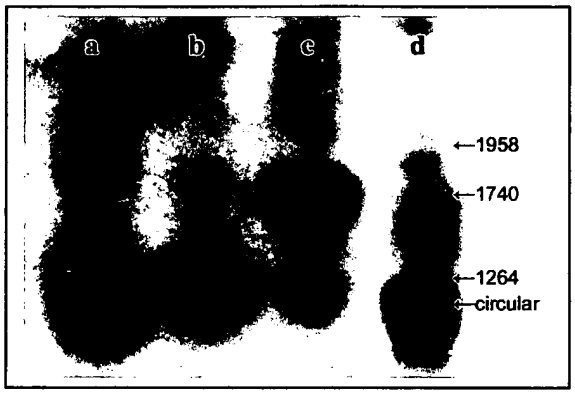


Figure 7



PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

ALTMAN, Daniel E.
KNOBBE, MARTENS, OLSON & BEAR, LLP
620 Newport Center Drive
Sixteenth Floor
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ETATS-UNIS D'AMERIQUE

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ATTORNEY RESPONSIBLE
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NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	28.05.2001
Applicant's or agent's file reference: NIH156.001VPC	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/04489	23/02/2000	24/02/1999

Applicant
THE GOVERNMENT OF THE UNITED STATES.....et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Hundt, D Tel.+49 89 2399-8042

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
NIH156.001VPC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/04489	23/02/2000	24/02/1999
International Patent Classification (IPC) or national classification and IPC A61K35/37		
Applicant THE GOVERNMENT OF THE UNITED STATES.....et al.		

1. This International preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03/08/2000	28.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Deck, A Telephone No. +49 89 2399 8432

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/04489

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-16 as originally filed

Claims, No.:

1-7 as originally filed

Drawings, sheets:

1/2-2/2 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-7
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-7
Industrial applicability (IA)	Yes:	Claims	1-7
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Concerning section V:

The following documents are referred to in this communication; the numbering will be adhered to in the rest of the procedure:

D1: BAUM BRUCE J ET AL: 'Re-engineering the functions of a terminally differentiated epithelial cell in vivo.' ANNALS OF THE NEW YORK ACADEMY OF SCIENCES., vol. 875, 18 June 1999 (1999-06-18), pages 294-300, XP000946428 Conference;Banff, Alberta, Canada; July 18-22, 1998, Technology, medicine, and materials. June 18, 1999 New York Academy of Sciences 2 East 63rd Street, New York, New York 10021, USA ISBN: 1-57331-194-4

D2: WO 97 45533 A (RUTHERFORD ROBERT BRUCE ;UNIV MICHIGAN (US); MOONEY DAVID J (US)) 4 December 1997 (1997-12-04)

D3: WO 99 01538 A (MASSACHUSETTS INST TECHNOLOGY) 14 January 1999 (1999-01-14)

D4: DELPORTE CHRISTINE ET AL: 'Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 94, no. 7, 1997, pages 3268-3273, XP002148673 1997 ISSN: 0027-8424 cited in the application

The document D1 reports a conference which was held on July 18-22, 1998, before the priority date of 24.02.1999 of the present application. Its whole content is therefore considered as part of the prior art relevant for examining the present invention.

1. Novelty

The present application does not meet the requirements of Article 33 (2) PCT because the subject-matter of claims 1 to 7 is not new for the following reasons.

The document D1 describes in detail the artificial salivary gland of the invention: a blind-end tube made of permeable, porous, biodegradable substrate, with a matrix capable of promoting the polarization of engineered cells, these cells being genetically engineered to be capable of unidirectional salt and water movement. The cells form a

monolayer on the internal surface of the support. Therefore, in view of the teaching in D1, the subject-matter of claims 1 to 6 is not new.

The documents D2 and D3 both describe an implantable device suitable for the regeneration of oral tissues amongst others. The device comprises a porous matrix (see D2, page 18, line 19 to page 19, line 26; page 20, lines 12 to 20; D3, page 20, lines 4 to 12) covered with cells like for example salivary secretory cells which implicitly produce water transport proteins (D2: page 21, line 4; D3: page 5, line 10 and page 20, lines 21-22).
The cells used in D2 and D3 can be transfected with a gene of interest in order to in vivo secrete a desired gene product (D2, page 16, lines 1 to 12; D3, page 22, lines 17 to 24).
Consequently the subject-matter of claims 1 and 7 is not new over D2 and D3.

2. Inventive step

Even if novelty were to be established, the present application would possibly not meet the requirements of Article 33 (3) PCT because the subject-matter would not involve an inventive step.
D1 clearly teaches the design of an artificial salivary gland claimed in the present invention. This device is useful in the treatment of salivary hypofunction.
Furthermore, an improvement of the device by increasing the salivary secretion via genetic engineering of the cells to produce additional water transport proteins would not be inventive as it can be derived from the teaching of D4 which describes successful hAQP1 gene transfer.
Finally, implantable devices as used in the invention have been described in detail in D2 and D3.

Therefore, it is presently not seen where an inventive step can be found in the present invention.

Concerning section VIII:

Although claims 1-5 have been drafted as separate independent claims, they appear to relate effectively to the same subject-matter and to differ from each other only with regard to the definition of the subject-matter for which protection is sought in respect of the terminology used for the features of that subject-matter. The aforementioned claims therefore lack conciseness. Moreover, lack of clarity of the claims as a whole arises, since the plurality of independent claims makes it difficult, if not impossible, to determine the matter for which protection is sought, and places an undue burden on others seeking to establish the extent of the protection.

Hence, claims 1-5 do not meet the requirements of Article 6 PCT.

In order to overcome this objection, it would appear appropriate to file an amended set of claims defining the relevant subject-matter in terms of a single independent claim in each category followed by dependent claims covering features which are merely optional (Rule 6.4 PCT).